The role of microorganisms in the nutrient cycling of subtropical savannah soils

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To my precious mother Anna and beloved sister Verena

Contents

Chapter 1

Summary

Soil is a complex ecosystem and constitutes an important carbon reservoir for the global nutrient cycling as it consists of $2.5*10^{12}$ t carbon in total (ocean: $1.3*10^{14}$ t carbon and atmosphere: $2.6*10^{12}$ t carbon). Apart from the supply of nutrients, soils support the development of a dense vegetation and ensure the production of crop yields and provide meadows for livestock (e. g. cows, goats and donkeys). In the temperate climate zone, soils recover fast after harvesting crops. Hence, soil fertility and crop yields are secured. However, about 40% of the continents are covered by nutrient poor semiarid soils with a low soil fertility and a low soil recovery capability. These mainly sandy soils are characterized by low water and nutrient contents, low water retention capacity and by an enhanced loss of macro- and micronutrients. At the same time, the majority of the world population depends on agricultural production on these nutrient limited soils. Nutrient cycling and the nutrient liberation in soils are mainly effected by the activity of the soil microbial community which is in turn varying with environmental parameters like pH, water and nutrient contents and temperature. Thus, central objectives of the present study were the identification of key organisms effecting nutrient cycling and the identification of environmental factors controlling and influencing biogeochemical processes.

In order to obtain insights into the nutrient cycling and its drivers, the activities of soil relevant exoenzymes and nitrogen transformation rates were investigated in Subsaharan arenosols. The breakdown of soil organic matter is initialized by exoenzymes. The present study revealed that the activities of the β-glucosidase, β-xylosidase, phosphatase and aminopeptidase determined in Namibian and Angolan savannah soils varied with the soil type, the land use type and the water availability. In sandy Subsaharan savannah soils the lower aggregate stability and the decreased nutrient and water availability reduced total bacterial cell numbers and thereby exoenzyme activity values. Consequently, microorganisms benefit from stable soil aggregates, nutrients and water in the darker loamy pristine soils of Kavango, pristine woodland and bushveld savannah soils of Mashare and in the peatland soils of Cusseque where high exoenzyme activity values were determined. Anthropogenic impact (i. e. fertilization, tillage, ploughing) and water stress after the dry season reduced total cell numbers of the soil microbial community and thereby activity values. Similar patterns of the land use type and the water availability effected nitrogen turnover rates of Mashare soils.

Highest ammonification and nitrification values were reached in the riparian woodland and bushveld soils after the rainy season. Total bacterial cell numbers and hence the nitrogen turnover rates decreased with increasing anthropogenic impact as well as the water stress caused by the dry season.

During a nutrient stimulation experiment, both land use type and water availability effected the active microbial community composition more than the amendment of nutrient solutions. Predominantly bacterial phyla and species adapted to nutrient limitation, water stress and heat like *Bacillus, Enterococcus* and *Arthrobacter* survived the environmental conditions in the sandy soils. However, after the prolonged dry season fast growing *Gammaproteobacteria* probably originating from the cattle and the local fauna dominated the active microbial community in the riparian woodland and bushveld savannah soils, while water stress decreased the amount of the metabolically active microorganisms so far. Similar activity patterns of subdivision 6 *Acidobacteria* after the addition of nutrients and water indicated fast response capability and dominance in the dry woodland soils. *Proteobacteria* (*Rhizobiales*) and *Actinobacteria* (*Arthrobacter* and *Rubrobacter*) prevailed in the riparian woodland and bushveld savannah soils after the rainy season. In contrast, anthropogenic impact such as the addition of fertilizers and ploughing decreased abundances of the *Proteobacteria* and *Actinobacteria,* whereas growth of the *Firmicutes* (*Exiguobacterium*) and to a lower percentage of subdivision 3 and 16 *Acidobacteria* was facilitated in irrigated fields. The results of the stimulation experiment also confirmed nutrient limitation in the examined soils. High abundances of nitrogen fixing microorganisms like diverse *Arthrobacter* species (*Actinobacteria*) and *Paenibacillus* (*Firmicutes*; associated with maize in the irrigated fields) and microorganisms performing nitrate ammonification (*Arthrobacter*) suggest nitrogen limitation. In addition, the clustering of soils supplemented with different phosphorus sources indicated phosphorus limitation in the Subsaharan savannah soils.

Although very little is known about subdivision 4 *Acidobacteria* and only two representatives have been validly described so far, this subdivision is detected in many different habitats like soils. Illumina high throughput sequencing confirmed an increased abundance of active subdivision 4 *Acidobacteria* in the riparian woodland and bushveld savannah soils after the rainy season. Even after the prolonged dry season the activity patterns were only slightly decreased in some soils, indicating that *Acidobacteria* subdivision 4 have an important role in Subsaharan savannah soils. For further analyses two new representatives were isolated from Namibian soil samples and characterized. Due to the morphological, physiological and molecular characteristics and a 16S rRNA gene identity of about 93% to

their next relative *Blastocatella fastidiosa*, the new isolates A22_HD_4H^T and Ac_23_E3^T were proposed as the new genus *Aridibacter* with the new species *A. famidurans* and *A. kavangonensis*, respectively. *A. famidurans* is well adapted to the nutrient limitation and the water stress in Subsaharan savannah soils. The formation of thick cell walls may enable this new subdivision 4 *Acidobacteria* to endure starvation and drought. *A. kavangonensis* is probably involved in the carbon cycling of subtropical savannah soils, since it is able to degrade complex substrates like starch and cellulose, two abundant polymers in soils. Therefore, both *A. famidurans* and *A. kavangonensis* are well adapted to the nutrient limitation and water stress in Subsaharan savannah soils.

This study revealed that the activity and the composition of the active microbial community in nutrient limited Subsaharan savannah soils are effected by the environmental parameters soil type, land type and water availability.

Chapter 2

General Introduction

2.1. Microorganisms and their relevance for nutrient cycling

Despite the research progress and importance in the clinical and biotechnological setting, rather little is known about microorganisms and their role in the environments. Microorganisms occur in various habitats like marine and limnic environments, polar deserts of Antarctica (Geyer *et al.,* 2014; Pointing *et al.,* 2009), hot springs (Brock *et al.,* 1969), wastewater (LaPara *et al.,* 2000), cave paintings (Schabereiter-Gurtner *et al.,* 2004), spacecraft assembly clean room environment (Vaishampayan *et al.,* 2013) and reach especially large numbers in soils (Janssen, 2006). One gram of soil contains up to 50,000 different species (Roesch *et al.,* 2007). This estimated number clearly exceeds the amount of all validly described species so far (~10500 species; Mora *et al.,* 2011).

Beside the meaning of the soil microbial community as a source of medical and biotechnological relevant secondary metabolites, the role of the microorganisms in the global nutrient cycling has high importance. The total microbial biomass on earth is estimated to consist of $4\text{-}6*10^{30}$ cells, reaching $0.25\text{-}2.5*10^{30}$ cells in the terrestrial subsurface and $2.6*10^{29}$ cells in soils (Whitman *et al.,* 1998). Therefore, microorganisms provide more biomass than eukaryotes on earth. Totally, the bacterial biomass of the world contains 350-550 Pg carbon (correlating to 60-100% of the carbon mass originating from plants), 85-130 Pg of nitrogen and 9-14 Pg of phosphorus (Whitman *et al.,* 1998). In addition to their important role as nutrient reservoir, microorganisms are the key players in the degradation and remineralization of organic matter, animal and plant residues and thereby the release of nutrients (Neumann *et al.,* 2014). Beside direct participation in the remineralization processes, microorganisms are located in the digestive system of degraders like ants and termites and support the degradation of complex compounds like cellulose by enzymatic activities (Rosenthal *et al.,* 2013). Without the activity of the soil microbial community in various remineralization processes, the world's reservoir of carbon would be fixed within 10-20 years to biomass and no further growth of microorganisms, plants and animals would occur (Fuchs *et al.,* 2007).

Despite the importance of the microorganisms in the global nutrient cycling, the controlling factors and key players of biogeochemical processes and thereby of nutrient release in soils are rather unknown. However, knowledge about these interdependencies

would support soil fertility even in low-fertility soils by the development of optimized land use management regarding microbial driven nutrient release.

2.2. Nutrient cycles

Nutrient cycling is the basis for life and growth on earth. Photosynthetic and chemoautotrophic producers (plants and microorganisms *Cyanobacteria* and *Betaproteobacteria*) transform inorganic carbon to organic material (Stanier, 1961), while consumers like animals and chemoheterotrophic microorganisms respire organic material as energy source within their metabolisms (Sterner *et al.,* 1992). However, the degradation of complex organic compounds and the liberation and remineralization of the macronutrients carbon, nitrogen and phosphorus are catalyzed by microorganisms. Due to their diversity and their adaptation to extreme conditions, microorganisms have evolved various strategies for nutrient transformations in several different ecological niches like the heterogeneous habitat soil. Here the microbial community supports and preserves nutrient release, soil fertility and plant growth by i.e. exoenzyme activities and nitrogen transformations.

2.2.1. Carbon cycle

The total living biomass on earth contains $2*10^{12}$ t carbon (Fuchs *et al.,* 2007), soil $2.5*10^{12}$ t carbon (Lal, 2004) and additional $2.6*10^{12}$ t carbon are available as CO_2 in the atmosphere (Fuchs *et al.,* 2007). CO_2 represents the central compound in the carbon cycle. Under anaerobic conditions some methanogens produce methane from acetate and CO_2 (Hook *et al.,* 2010). In contrast, methanotrophs transform methane to CO_2 in the presence of oxygen (Hanson *et al.,* 1996). Photosynthesis performed by plants and autotrophic microorganisms (*Cyanobacteria*, *Betaproteobacteria*) fixes CO_2 to organic material. During the anoxygenic photosynthesis CO_2 is transformed to organic material without the liberation of oxygen. Under aerobic conditions plants and *Cyanobacteria* provide oxygen derived from water (Nogales *et al.,* 2012; Nagashima *et al.,* 2012). Consumers respire the liberated oxygen and carbon sugars like glucose and fructose in their metabolism. However, wood and grass biomass consist of nearly 75% of heavy degradable complex carbon compounds like cellulose, hemicellulose, starch, pectin, arabinogalactane and of 20% of lignin and lignan (Sjöström, 1993). In contrast to animals, microorganisms are able to degrade these complex compounds to remineralize carbon by enzymatic activity (Gupta *et al.,* 2012) (Fig. 1). For this reason microorganisms often colonize digestive systems of ants and termites as symbionts.

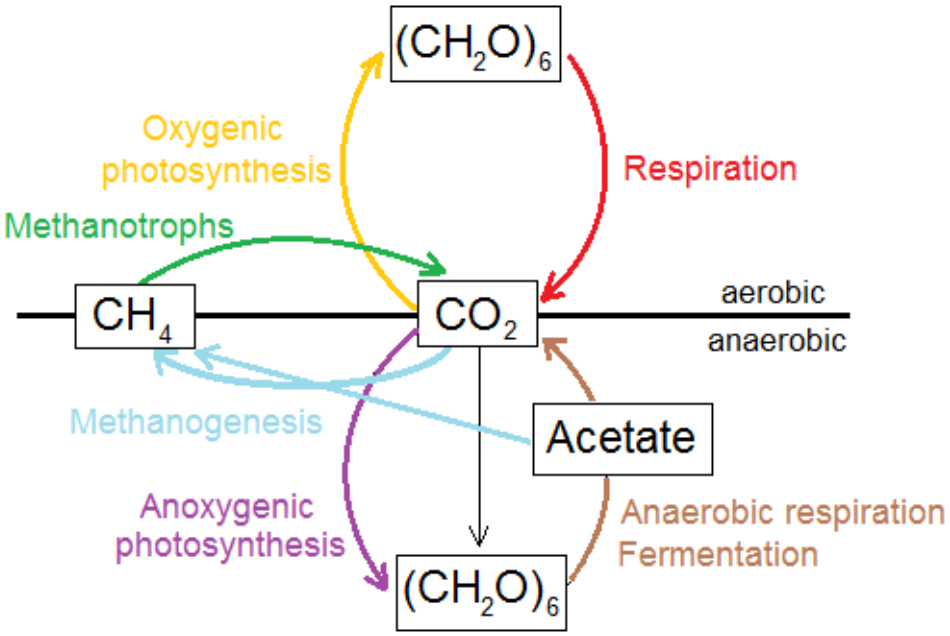

Figure 1: Carbon cycle: Transformation of carbon compounds under aerobic and under anaerobic conditions. Aerobic carbon transformations: red, respiration; yellow, oxygenic photosynthesis and green, methanotrophs. Anaerobic carbon transformations: brown, anaerobic respiration/fermentation; blue, methanogenesis and purple, anoxygenic photosynthesis. Graphic compiled by author.

2.2.2. Nitrogen cycle

Nitrogen constitutes up to 15% of the living bacterial biomass (Vrede *et al.,* 2002). However, 99% of the total nitrogen amount on earth is available as nitrogen gas (Brandes *et al.,* 1998). Only a small percentage of the molecular nitrogen is fixed or transferred into the nitrogen cycle by nitrogen fixers like *Rhizobiales*, *Arthrobacter* and *Frankia*. Therefore, the liberation of nitrogen from biomass is essential for a nutrient regeneration further growth and for the preservation of life (Fuchs *et al.,* 2007). Ammonium and nitrate are the most important nitrogen sources and can be assimilated by the majority of the soil organisms. In soils nitrate is easily washed out and ammonium is adsorbed to soil particles (Blume *et al.,* 2010). Therefore, nitrogen may become scarce and thus is the prevailing limited nutrient in the soil environment. Microorganisms have evolved several pathways in order to counteract nitrogen limitation of growth. The enzyme aminopeptidase and the ammonification process liberate amino compounds from plant and animal residues (Omar *et al.,* 1999). Under aerobic conditions nitrification transforms ammonium to nitrite and nitrite to nitrate (Ward, 1996). Nitrate is either directly assimilated or used as an alternative electron acceptor under anaerobic conditions (Fuchs *et al.,* 2007). The natural content of soil nitrogen can also be increased by the activity of anaerobic ammonia oxidizers like the planctomycete *Brocadia*. This bacterium is able to perform anaerobic ammonia oxidation and transforms ammonia and

nitrite to nitrogen (Madigan *et al.*, 2007). In turn, the increased amounts of nitrogen are fixed by nitrogen fixers like *Rhizobiales*, *Arthrobacter* and *Frankia* (Fig. 2).

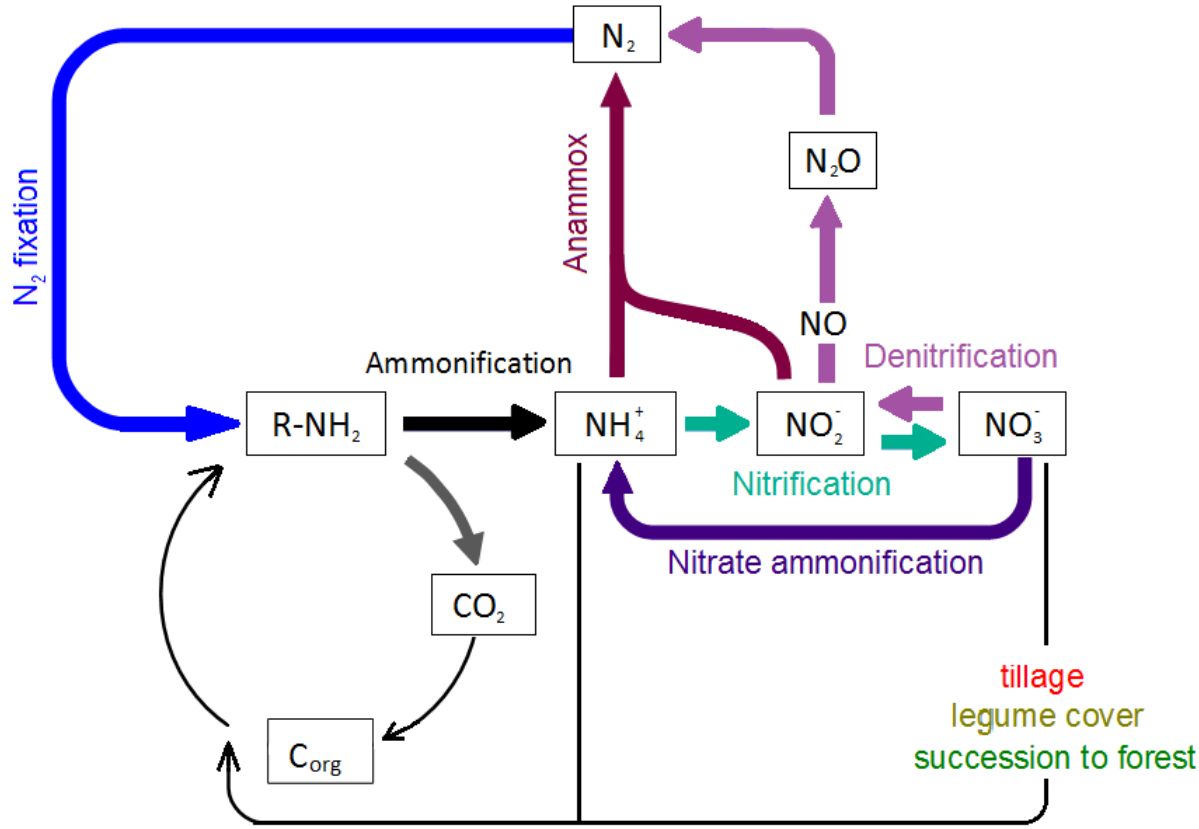

Figure 2: Nitrogen cycle and transformation pathways. Blue, nitrogen fixation; black, ammonification, bourdeaux, anammox; turquoise, nitrification; purple, nitrate ammonification; lavender, denitrification. Ammonia and nitrate as organic compounds.
Graphic compiled by Jörg Overmann modified by the author.

2.2.3. Phosphorus cycle

Beside nitrogen, phosphorus is also often limited in soil (Vitousek *et al.*, 2010). In soils phosphorus is part of insoluble complexes like apatite and heavy metal complexes and therefore unavailable for the soil microbial community (Belnap, 2011). However, phosphorus is an important nutrient in the biogeochemical cycle since it is part of nucleic acids and of the energy transporting component ATP (Madigan *et al.*, 2007). Therefore, the liberation of this macronutrient from organic residues is essential for living and growing organisms. Microbial phosphatase liberates phosphate by the hydrolysis of phosphate ester bonds from soil organic matter, plant and animal residues (Spohn *et al.*, 2013). Then, the liberated phosphate is either assimilated in the biomass or bound in insoluble soil complexes.

2.3. The ecosystem soil and its microbial community

The ecosystem soil constitutes a highly diverse habitat for plants, animals and in particular microorganisms. According to 16S rRNA analysis, *Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes* and *Firmicutes* represent typical bacterial phyla in soils with decreasing abundance values according to the order (Janssen, 2006). Moreover, the phyla *Acidobacteria* and *Proteobacteria* reached 5-46% and 10-77% of the soil microbial community, respectively (Janssen, 2006). In contrast, the composition of the culturable bacterial fraction mainly consisted of *Arthrobacter* (5-60%), *Bacillus* (7-67%), *Pseudomonas* (3-15%), *Agrobacterium* (up to 20%), *Alcaligenes* (2-12%), and *Flavobacterium* (2-10%) (Alexander, 1977). This discrepancy of culturable microorganisms and the real composition of the soil microbial community reveals the missing knowledge about microorganisms in the heterogeneous environment soil.

Soils consist of a predominant solid phase surrounded by variable amounts of gases and water. Microorganisms are either attached to soil particles, located in the water surrounding the soil particles or live inside soil aggregates (Huang *et al.,* 2002). The interaction of different soil particles and the absorption to each other results in the formation of soil aggregates. According to their size soil particles can be grouped into sand (63-200 µm), silt (2-63 µm), clay (under 2 µm) and loam (mixture of sand, silt and clay at equal amounts). Due to differences in physical, chemical and biological characteristics soils are vertically distributed in horizons (Blume *et al.,* 2010). The abundances and the composition of the microbial community vary with the vertical distribution and the soil characteristics. The highest microbial activity is located in the upper layer, the O-horizon (i. e. litter), with a high content of organic compounds (Ekelund *et al.,* 2001). The subsequent A- (humic upper) layer still contains various representatives of the soil microbial community, while the deeper layers, the B- (weathered subsoil) and C-horizon (mineral ground), show decreased microbial abundances and organic matter contents. The total organic matter content of the horizons distinguishes soils. An organic content less than 20% within the O- and A-horizons characterizes mineral soils like sandy arenosols of Namibia and Angola and an organic content above 20% organic soils like the peatland soils in the Angolan highlands (Tate, 2000).

Microorganisms are either located in the rhizosphere of plants or in the bulk soil. Depending on the influence of the vegetation and the physical soil characteristics the composition of the microbial community varies. Representatives of *Rhizobium, Bradyrhizobium, Agrobacterium*, (all *Rhizobiales*/*Proteobacteria*) and of plant associated

Actinobacteria like *Frankia* dominate in the rhizosphere. Moreover, the presence of root exudates as an additional nutrient source effects the bacterial community composition (Kent *et al.,* 2002; Lynch *et al.,* 2001) as well, i. e. *Paenibacillus brasiliensis* is affiliated with the maize rhizosphere (von der Weid *et al.,* 2002). In contrast, *Acidobacteria*, *Firmicutes* and other *Proteobacteria* prevail in the predominantly oligotrophic bulk soils. Soil microorganisms are exposed to the various and challenging conditions in soils like the water stress and nutrient limitation and have evolved several different adaptation mechanisms, i. e. exo- or endospore formation (Chen *et al.,* 2014), optimized DNA reparation systems (Mongodin *et al.,* 2006), high affinity exoenzymes, phosphorus stress response (Makino *et al.,* 1993), nitrogen fixation and even photosynthesis. Hence, soil is an adequate study site for isolating new species and examination of nutrient cycling under varying conditions.

2.4. Subsaharan Namibian and Angolan savannah soils and microbial community

2.4.1. Soil types

In this study soil bacteria were investigated in arenosols sampled in the Bié region of the Angolan highland, in the Okavango basin near the Namibian village of Mashare and in the Kavango region near the Mutompo village in the north-east of Namibia.

Arenosols are sandy soils dominated by coarse particles and are characterized by low nutrient and water contents due to a low water retention capacity (van Wambeke, 1992) (Fig. 3 A). Aggregates in sandy soils are easily disrupted by animal and human activities. Hence, the amount of microbial microhabitats is limited. Therefore, microorganisms only colonize the surface of the sandy particles and the water surrounding the particles. In sandy soils microbial abundances and hence the activity levels (i. e. respiration and exoenzyme activities) are low compared to soils with defined horizons and stable soil aggregates.

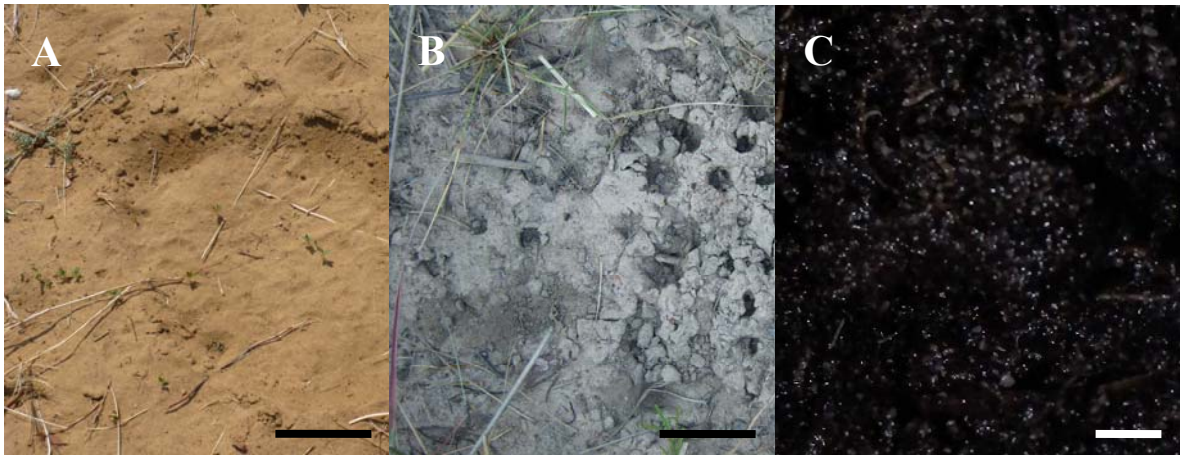

Figure 3: Soil types of the Subsaharan savannah soils. **A.** Kalahari Sands dominating the Namibian and Angolan sampling sites **B.** loamy sands in the river basins and pristine soils and **C.** peatland soils of the Angolan highlands. Bars in panel **A.** and **B.**, 5 cm and in panel **C.**, 1cm.

However, even arenosols vary in physical, chemical and biological conditions. Soil organic matter content stabilizes soil texture, improves the water retention capacity and provides nutrients and water for the microbial community. Additionally, the improved water retention capacity prevents the leaching of free living soil microorganisms. In the old flood plains soils of the Okavango basin of Mashare (Fig. 3 B) and the peatland soils of the Angolan highland (Fig. 3 C) soil organic matter contents are increased compared to the sandy arenosols.

2.4.2. Land use types

Anthropogenic impact, i. e., ploughing, cultivation, harvesting and fertilization significantly impacts soil conditions (Tilman *et al.,* 2001). Agricultural activities lead to changes of the soil environments, destroy soil aggregates as well as microhabitats and compact the bulk soil. The addition of fertilizers alters the natural pH and changes the dissociation and solubility of enzymes, substrates, cofactors, stability of protein structures, organic xenobiotics and metals, which might act as inhibitors. Metals added in fertilizers mask catalytically active groups, denaturize or compete with cofactors (Baldrian *et al.,* 2008; Baldrian *et al.,* 2009; Burns & Dick, 2002; Effron *et al.,* 2004; Gianfreda *et al.,* 1996; Kandeler *et al.,* 1999). Hence, some anthropogenic treatments on soils reduce the microbial abundances and negatively influence the nutrient turnover of carbon, nitrogen and phosphorus (Waldrop *et al.,* 2000; Cleveland *et al.,* 2003).

Pristine soils like the riparian woodland (Fig. 4 A), bushveld savannah (Fig. 4 B) and the Angolan peatland soils are nearly undisturbed by anthropogenic impact. However, the percentages of pristine areas around the settlements in Namibia and Angola are decreasing. While the bushveld savannah soils are mainly used for meadows, natural woodlands have nearly vanished in the north-east of Namibia. Only two small woodland areas exist near the village of Mashare. The leaching of nutrients in fields and the resulting decrease of harvest crops lead to a continuous conversion of pristine land to agriculturally used regions. In the examined area land is cultivated by the principle of dry agriculture (Fig. 4 E/F).

The predominant crop is mahangu/millet, which is directly planted around the villages. After sowing the fields are naturally watered and wild animals are kept away by fences or people guarding the fields. The cultivated areas are continuously planted. When harvest crops decrease, the settlements move and search for fertile farmland. For recovery of soil fertility the former fields are left behind as fallow. Beside dry fields, irrigated fields are localized in the Namibian basin of the Okavango river (Fig. 4 D). These fields are artificially irrigated with river water and fertilized to optimize harvest crop yields of maize and wheat in the old flood plain soils.

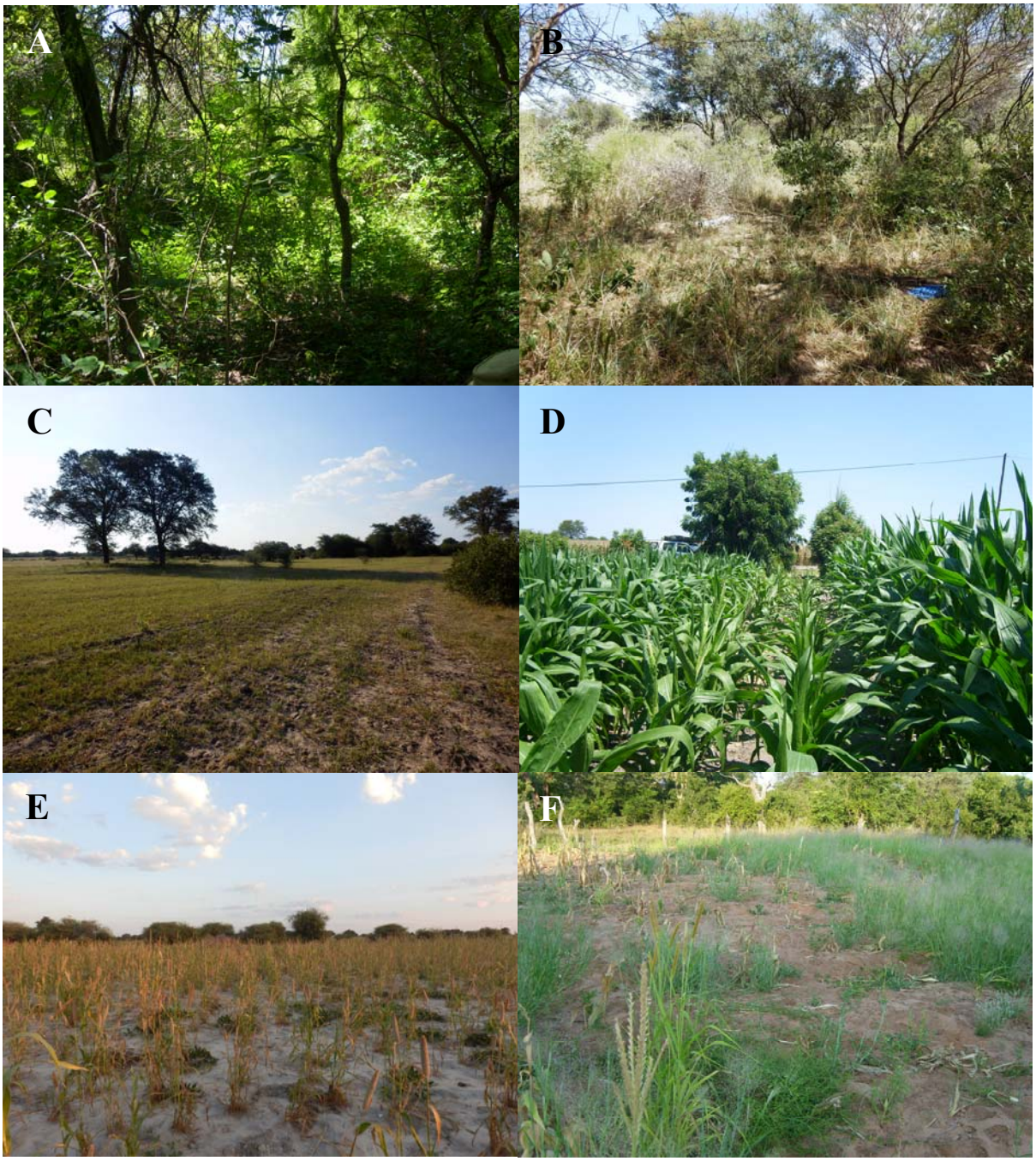

Figure 4: Land use types near the village Mashare (Namibia) in the basin of the Okavango river. **A.** riparian woodland, **B.** bushveld, **C.** fallow, **D.** irrigated field and dry fields in **E.** the old flood plains of the Okavango river and **F.** in the Kalahari Sands dunes. Irrigated fields are planted with maize (*Zea mays*) and dry fields predominantly with mahangu/millet (*Penisetum glaucum*).

2.4.3. Climate and seasonal variability of water availability

Beside the soil type and land use type, the water content is a soil characterizing environmental parameter and varies with the season in subtropical savannah soils of Namibia and Angola. All Namibian sites are characterized by semiarid and the Angolan sites by temperate tropical climate of the southern hemisphere. Rainfall occurs during the summer months between November to March and is followed by the dry season from April to October. Average annual precipitation in the Angolan highlands is 1111 mm/m and exceeds that of the Namibian sampling sites at Kavango (544 mm/m) and Mashare (595 mm/m) (AQUASTAT, 2014). The annual mean temperature in the Kavango and Mashare region (21.9°C and 22.3°C, respectively) slightly exceed the value of 19.2°C at the Angolan sampling site (AQUASTAT, 2014).

Variations in water availability directly influence the composition and the activity of the soil microbial community. Water stress and the presence of high soil temperatures as caused by the dry season decrease microbial diversity (Sheik *et al.*, 2011). Activity and therefore nutrient cycling directly correlates to the amount of bacteria (Manzoni *et al.,* 2012). Therefore, the abundances and the activity of the soil microbial community decreases with decreasing water availability as determined for soil respiration in hardwood forests (Davidson *et al.,* 1998). Only bacteria adapted to drought, UV radiation and heat survive the months of drought.

2.5. Analysis of soil microbial diversity and activity

2.5.1. Microbial diversity

Quantification of microbial abundances is the basis to understand microbial community structure and population dynamics under different environmental conditions. Methods for quantifying the microbial community are either culture dependent or culture independent. The determination of the most probable number (MPN) with subsequent physiological tests for identification of the obtained cultures belong to the culture-dependent methods (Alexander, 1965). These methods rely on the viability and growth of microorganisms on culture media. Only a small percentage of the soil microbial community is culturable and the majority of the microorganisms is not detected (Alexander, 1974). Furthermore, these methods are time consuming and afford a high variety of media and consumables.

Currently, the microbial diversity is typically determined by culture-independent methods. Most culture independent methods, i. e. DGGE (denaturing gradient gel electrophoresis), qPCR (quantitative polymerase chain reaction) and clone libraries, are based on the analysis of the 16S rRNA gene. The combination of conservative and highly variable

motifs makes the 16S rRNA gene an optimal universal marker for community analysis. Real-time PCR (qPCR) provides high sensitivity, is quantitative (Walker, 2002) and less laborious than whole cell applications (Cavicchioli *et al.,* 2003) like the FISH techniques (fluorescence in situ hybridization) (Labrenz *et al.,* 2004). After the extraction of DNA or RNA, a polymerase chain reaction (PCR) amplifies with (specific) primers the respective sequences. The gained PCR products are further separated by finger printing methods like DGGE or T-RFLP (terminal restriction fragment length polymorphism) and identified by Sanger sequencing. The Sanger method belongs to the first generation sequencing technology. Sanger determines up to 1000 bp long sequences with a high efficiency and low sequencing errors by incorporation of selective chain terminating nucleotides during the sequencing. But the Sanger method requires DNA of one organisms per one run and is therefore improper for the analysis of co-cultures or environmental mixture samples.

Further development resulted in several new next generation sequencing technologies. The great advantage of all next generation sequencing methods is the high amount of generated sequences and direct determination of the microbial community composition in the samples (Metzker, 2009). After the nucleic acid extraction, the sequence libraries are prepared. All next generation sequencing methods are based on the multiplexing of templates on a solid phase during the sequencing. But the existing technologies like 454 (Roche), Illumina (Solexa) and PacBio (Pacific Biotechnologies) differ in the length and amount of the generated sequences and the sequencing error. The 454 sequence system (Roche) fixes a template to a bead. The beads are chemically cross-linked in wells. Clonal amplification occurs in microreactors in an emulsion and the sequencing process generates up to 1 billion sequences with 300-800 bp length and a consensus accuracy of 99.997%. But the presence of homopolymers of the different bases challenges the 454 sequencing systems resulting in overexposure and wrong signals (Metzker, 2009). Within the Illumina (Solexa) technology fragmented DNA is ligated to a solid phase, followed by a bridge amplification to generate DNA clusters. One Illumina HiSeq run generates 20 billion reads per flow cell with an average fragment size of 50-300 bp and a consensus accuracy of 99.999% (Metzker, 2009). Because of the high amount of high quality sequences with relatively low cost the Illumina technology was chosen as the appropriate method for the analysis of the soil microbial community composition in Subsaharan soils.

Culture-independent methods have facilitated the study of environmental communities without cultivation. These methods provide a possibility to quantify bacterial diversity in natural environments. However, only a combination of culture-independent and culture-

dependent approaches provides the understanding of the interaction between the microorganisms and their natural environment.

2.5.2. Microbial activity

The examination of microbial activity patterns under different environmental conditions enables the identification of the main controlling factors. Microbial activity in soils is either directly measured by the determination of soil respiration, CO_2 flux, nitrogen turnover and exoenzyme activities or by monitoring the gene expression with RNA-SIP (stable isotope probing), RNA-DGGE, microarrays, qPCR or RNA sequencing.

Central parts of nutrient cycling are effected by exoenzyme activities (Dick *et al.,* 1988; Concilio *et al.,* 2006; Gupta *et al.,* 1988; Kandeler *et al.,* 1999; Acosta-Martínez *et al.,* 2007). Exoenzymes are important indicators of soil fertility because they show a rapid change and high sensitivity towards changes of the environment (Bandick *et al.,* 1999). They are secreted by microorganisms (Ladd, 1978), but also by plants and animals (Tabatabai, 1994) to degrade complex substrates and regain nutrients (Harder *et al.,* 1983; Sterner *et al.,* 2002; Allison, 2005). The MUF/AMC assay (methylumbelliferone/aminomethylcoumarine) determines exoenzyme activities by the release of fluorophores from the respective substrate analogues. The amount of liberated fluorophore directly correlates to microbial exoenzyme activities (Weintraub *et al.,* 2007).

Ammonification and nitrification as considerable nitrogen liberating processes directly correlate to the microbial community and are effected by different environmental parameters like soil type, land use type and water availability. Gross ammonification and nitrification rates are determined by the Pool Dilution Technique (PDT). The natural $^{14/15}N$ ratio is artificially increased by the amendment of ^{15}N marked salts to soils. The following analysis of the $^{14/15}N$ ratio changes over time at the IRMS (isotope ratio mass spectrometry) allows the calculation of ammonification and nitrification rates (Barraclough, 1995; Barraclough & Puri, 1995; Booth *et al.,* 2005).

Beside the composition of the active microbial community, Illumina high throughput total RNA sequencing enables the analysis of the mRNA. Hence, regulated genes and activated metabolisms pathways under certain conditions are identified. Adequate identification of mRNA gained from environmental parameters requires appropriate mRNA banks. However, the existing mRNA banks constitute of sequences mainly deriving from organisms affiliated with the human microbiome. Therefore, the correct assignment of the environmental mRNA sequences is complicated.

2.6. Aims and background information of the present study

The present study was part of the BMBF (Bundesministerium für Bildung und Forschung, Germany) funded projects BIOTA (Biodiversity Monitoring Transect Analysis in Africa) and TFO (The Future Okavango). Within the BIOTA and the TFO projects the interdependencies of environmental parameters and the microbial community and microbial processes were analyzed. The understanding of the microbial role in the nutrient cycles provides science based knowledge for the development of improved sustainable land use management concepts. These concepts supply on the one hand the preservation of the unique biodiversity of the Okavango river basin and on the other hand the food supply of million of people.

Our knowledge about nutrient cycling is still scarce. However, soil fertility and thereby crop yields are dependent on the efficiency of nutrient cycling which are predominantly effected by the soil microbial community. But rather little is known about the controlling factors of microbial activity and the interdependencies of environmental factors and the soil microbial community.

In order to elucidate the role of the soil microbial community in the nutrient cycles of subtropical savannah soils, exoenzyme activities were investigated in 77 Namibian and Angolan soil samples differing in soil type, land use type and water availability and the nitrogen turnover rates were examined in 20 Namibian soils differing in land use type and water availability. In the present study, the active soil microbial community was manipulated by the addition of soil relevant nutrients, in order to identify limiting nutrients, important metabolic pathways of soil microorganisms and potential key players in the nutrient cycles of subtropical savannah soils like the soil dominating *Acidobacteria.*

Acidobacteria are ubiquitous and highly abundant in numerous habitats. This phylum constitutes up to 77% of the microbial community in soils. Due to their capability to degrade complex compounds and to adapt to extreme environmental conditions *Acidobacteria* might have an important role in Namibian and Angolan arenosols. Therefore, the identification and characterization of two novel representatives of the ubiquitous but poorly understood *Acidobacteria* enable the examination of these dominating soil bacteria.

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Chapter 3

Variations and controls of hydrolytic exoenzyme activities in Subsaharan savannah soils

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3.1. Contribution of the authors

The exoenzyme measurements obtained in the soil samples of Mutompo where examined by Delita Zul, while Katharina Huber determined the activities of the exoenzymes β-glucosidase, β-xylosidase, phosphatase and aminopeptidase in the 23 Namibian soils of the Mashare campaign 2011, in the 25 soils of the Mashare campaign 2012 and in the 19 Angolan soils of the Cusseque campaign 2012. Katharina Huber calculated all exoenzyme activities of this study and conducted the figures. The multivariate statistical analysis were investigated by Johannes Sikorski. Katharina Huber and Jörg Overmann wrote this article together.

3.2. Abstract

Hydrolytic exoenzymes are key agents of nutrient liberation and retention in soils. In order to determine the specific role of exoenzymes under nutrient-poor conditions in Subsaharan savannah ecosystems, the variability and controls of β-glucosidase, β-xylosidase, phosphatase and aminopeptidase activities were investigated in 77 Namibian and Angolan arenosols differing in climatic and physicochemical conditions and land use. Potential exoenzyme activities V_{max} were generally low [$\leq$ 2 µmol $(g_{DW}\ h)^{-1}$] in the nutrient poor Kalahari sands but also in the darker loamy sands of dry farming fields, fallows, anthropogenic grasslands, as well as certain bushvelds and woodlands. Significantly higher activities [V_{max} up to 16.3 µmol $(g_{DW}\ h)^{-1}$] were determined in loamy sands of dry bushvelds and woodlands but only very rarely in agricultural fields or anthropogenic grasslands. The highest exoenzyme activity values [56.3-122.8 µmol $(g_{DW}\ h)^{-1}$] were detected for aminopeptidase in the pristine and horticulture peatland soils of Cusseque (Angola). In contrast to V_{max}, values of $K_m + S_n$ (the sum of the half saturation constant plus the concentration of natural substrate present) were more similar between different soil and land use types and in most samples were highest for phosphatase (up to 466 µM). Maximum values of $K_m + S_n$ were between 461 and 565 µM for aminopeptidase and determined in Angolan peatlands and one fallow soil at the Okavango river. Redundancy analysis of climatic factors, soil physicochemical parameters and land use types identified bacterial cell numbers, organic carbon and anion content as the main determinants for the activity of the exoenzymes which however responded differently to soil C/N ratios and pH.

3.3. Introduction

Arenosols cover about 10% of the land surface of the Earth. As deep aeolian Kalahari sands, arenosols prevail on the central African plateau between the equator and 30° southern latitude and cover 51% and 31% of the land surface of Angola and Namibia, respectively (Hartemink & Huting, 2007). These soils are characterized by a low water-holding capacity, low cation exchange capacity, a low organic matter content of < 1%, which in turn cause a low soil nutrient content and fertility (van Wambeke, 1992; Pröpper *et al.*, 2010). Soils on Kalahari sands are particularly sensitive to changes in land use. Typically, rapid decomposition of soil organic matter and a decrease in cation exchange capacity follows the conversion of pristine land into rain-fed agriculture fields, leading to nitrogen and particularly phosphorus deficiency of cropped fields (Dougill & Thomas, 2004; Pröpper *et al.* 2010; Solomon *et al.*, 2007). A better understanding of the functional basis of nutrient cycling in arenosols is therefore essential for their sustainable management in the future.

Similar to soils in more humid regions (Paul, 2007), the major fraction of carbon and nitrogen in arenosols resides in the organic macromolecular fraction (Gröngröft *et al.*, 2013). Although dryland soils, in contrast to their mesic counterparts, contain phosphorus mostly in the form of inorganic complexes, the latter are biologically largely unavailable (Belnap, 2011). Similar to other soils, nutrient regeneration in African savannahs is therefore expected to be determined by the degradation of soil organic matter, but the mechanisms of degradation and their environmental controls in drylands are so far little understood (Sinsabaugh *et al.*, 2008).

In soils, hydrolytic enzymes control the degradation of organic matter and nutrient regeneration (German *et al.*, 2012; Marx *et al.*, 2001; Sinsabaugh *et al*., 2008). Phosphatase (PHO) in soils originates from bacteria and fungi (Tabatabai, 1994), represents the best studied enzyme of phosphorus mineralization and is thought to be most important for P-supply because of its broad substrate spectrum (Duhamel *et al.*, 2011). In fact, up to 87% of organic phosphorus compounds in dryland soils are hydrolyzable by phosphatases (Belnap, 2011). PHO activities are subject to feedback control and decrease with higher concentrations of inorganic phosphate (Sinsabaugh *et al.*, 2008). β-glucosidase (BG) hydrolyses different polysaccharides such as cellobiose, triose and β-glucosides, whereas β-xylosidase (BX) controls the breakdown and decomposition of hemicelluloses. These enzymes participate in cellulose and hemicellulose degradation and exhibit elevated activities in systems receiving larger amounts of plant litter (Boschker & Cappenberg, 1998) but decreased activities in the presence of readily assimilable organic compounds (Rinkes *et al.*, 2013). Leucine

aminopeptidase (LAP) hydrolyses the most abundant protein amino acid from the N-terminus of polypeptides. LAP activity can decline upon N amendment (Alster *et al*., 2013). Accordingly, activity patterns of these four exoenzymes not only provide information on the dominant pathways of organic matter mineralization and nutrient regeneration (Krämer & Green, 2000) and on the composition and source of the organic substrates (Boschker & Cappenberg, 1998), but can also yield insights into the environmental controls and feedbacks of the underlying microbial processes (Delgado-Baquerizo *et al*., 2013).

Whereas changes in soil management result in rapid changes in exoenzyme activities (occurring within one year in temperate zones), the effects on microbial biomass and N-mineralization occur more slowly (several years), and changes in organic carbon content occur only over decades (Kandeler *et al*., 1999b). This is also true for Subsaharan grasslands (Lobe *et al*., 2001; Preger *et al*., 2010). Because of their rapid response, exoenzyme activities thus represent sensitive indicators for the effect of land management on the quality of agricultural soils (Bandick & Dick, 1999; Kandeler *et al*., 1999b).

In order to obtain insight into the drivers of nutrient cycling in nutrient poor Savannah arenosols, the activities of β-glucosidase, β-xylosidase, phosphatase and leucine aminopeptidase together with a large number of environmental parameters were studied in the Western part of the central African plateau. The 77 Angolan and Namibian sampling sites covered a variety of different soil and land use types as well as climatic and physicochemical conditions and are under particular pressure due to the high density of the human rural populations.

3.4. Materials and Methods

3.4.1 Study site

A series of 77 soils had developed on Kalahari sands and were located in three different regions of Angola and Namibia (Fig. 1). The study sites differed in soil type, land use as well as climatic conditions (compare Suppl. Table 1). Within the Angolan highlands, soil samples were collected at Cusseque in the Bié region (13°42´8.0´´S, 17°4`35.0´´E; 1,548 m above sea level; Fig. 1 A, B). At this site, *Acacia nigrescens*, *Baikea plurijuga*, *Terminalia sericea*, *Combretum collinum* and *Feijao frade* prevailed in forests and bushlands. The two other sites are located in the northeast of Namibia. Soils in the Okavango river valley were sampled near Mashare (17°53´40.9´´S, 20°10´39.6´´E; 1,070 m above sea level; Fig. 1 A, C). While *A. nigrescens* and *Peltophorum africanum* prevailed in pristine woodlands at the riverside, *A. erubescens and A. luederitzii* dominated the more distant thornbush savannah. The third sampling site is located in the Kavango region near the village of Mutompo (18°18´38.3´´S, 19°17´49.6´´E; 1,110 m above sea level; Fig. 1 A, D). The forest savannah around the Mutompo village represent Kalahari dry woodlands consisting of different *Combretum species*, *T. sericea*, *Burkea africana*, *Eragrostis echinochloides* and *Guibortia colophosperma*. Dominating crop plants at all three sites were *Sorghum bicolor* (sorghum, milo), *Pennisetum glaucum* (pearl millet, mahangu), *Zea mays* (maize), and *Vigna unguiculata* (cowpea).

The Namibian sites have a hot semiarid climate and the Angolan site a temperate tropical climate with dry winters. Rainfall occurs during the summer months between November to March and is followed by a dry season from April to October. Average annual precipitation in the Angolan highlands is 1,111 $mm \cdot m^{-1}$ and significantly exceeds that of the Namibian sampling sites at Mutompo (544 $mm \cdot m^{-1}$) and Mashare (595 $mm \cdot m^{-1}$). The annual mean temperature in the Mutompo and Mashare region (21.9°C and 22.3°C, respectively) exceeds the value of 19.2°C at the Angolan sampling site (AQUASTAT, 2014). Ten different sites were sampled at Mutompo in March 2007. 23 and 25 different soils of the Okavango river valley at Mashare were collected in October 2011 and in March 2012, respectively (Suppl. Table 1). In March 2012, 19 samples were collected at Cusseque within the upper part of the Okavango catchment. Similar values for precipitation and temperature were observed in each region between the rainy season 2006/2007 and that of 2011/2012 which renders the data from the two sampling campaigns comparable.

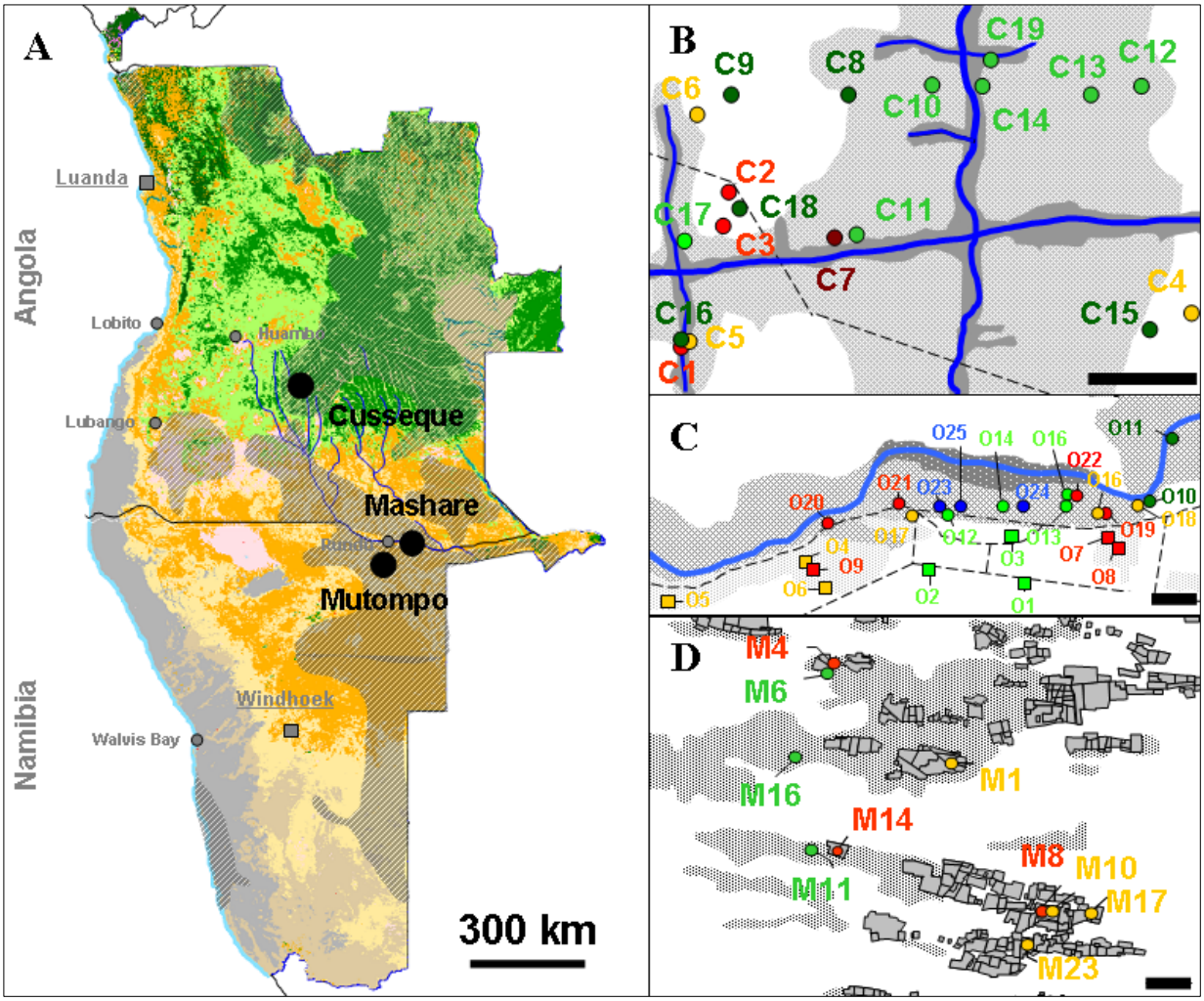

Figure 1: Location of the study sites on the central African plateau. **A.** Overview showing the three main sampling sites at Cusseque, Mashare and Mutompo. Green colors designate forested areas (dark green, evergreen; light green, open deciduous forest), orange and yellow colors shrublands and grasslands, respectively, and grey areas deserts. The hatched area indicates the geographic distribution of Kalahari sands (based on Hartemink & Huting, 2007). **B.** Detailed map of Cusseque sampling site. Dark grey shading depicts wetlands of the Cusseque river and its tributaries, light grey shading the slopes of the river valley. **C.** Detailed map of Mashare sampling sites. Dark grey shading depicts wetlands of the Okavango river, grey shading old flood plains and light grey shading agriculturally used areas in the Kalahari sands. **D.** Detailed map of Mutompo sampling site. Grey polygons denote agricultural fields, grey shading *Acacia* thornbush vegetation of the interdune valleys, and white areas the open tree savannahs on Kalahari sands. Sampling points in panels **B.** through **D.** indicate pristine woodlands (dark green), grasslands and bushveld (light green), dry agriculture fields (red), irrigated fields (blue), and fallows (yellow). In panel **C.** dots indicate sites located in old floodplains and squares the sites in sandy areas. Dashed lines, roads. Bars in panels **B.** through **D.**, 1 km.

3.4.2 Soil sampling and characteristics

After removing the litter layer that was present on top of some woodland and bushland soils, the upper 10 cm of each soil were collected along two perpendicular transects that each encompassed 5 sampling spots spaced 5 m apart. The intersection was sampled once, yielding a total of 9 samples per location, which were pooled and homogenized. The pooled soil samples were kept at 4°C during the transport to the laboratory.

Soil temperature was directly measured in the field in the top 10 cm of the soils (Checktemp 1 thermometer; Hanna Instruments, Kehl). The other physicochemical parameters were investigated using aliquots of the mixed soil samples. Dry weight was measured after drying 1 g of soil at 80°C for 72 h and pH values determined in distilled water as well as in 10 mM $CaCl_2$ solution. The total carbon and total nitrogen contents were investigated by dry combustion analysis (Vario Max; Elementar Analysensysteme GmbH, Hanau, Germany). The organic carbon content was analyzed as the loss in carbon upon combustion of soil samples at 450°C for 16 h. A double-lactate extraction was employed to quantify P concentrations and the amounts of K^+, Na^+, Mg^{2+}, Ca^{2+}, $Fe^{2+/3+}$, Cl^-, NO_3^-, NO_2^-, SO_4^{2-} and HCO_3^- were determined in aqueous extracts of the soils [1:1 (w/v)] (Herpel, 2008). Sampling of Mashare soils before the rainy season was conducted for an exemplary comparison of exoenzyme activities at this single site. Since a parallel sampling of the other two study areas was not feasible before the rainy season, only the parameters TCN, pH and temperature were measured in October 2011 at Mashare.

3.4.3 Total cell counts

Soil samples were fixed in glutaraldehyd fixation solution [1% (v/v) in 5.5 mM MES (2-(N-morpholino)ethanesulfonic acid) buffer, pH 6.0]. 50 µl of the soil slurries were mixed with 450 µl of methanol and 1000 µl of MOPS buffer [10 mM 3-(N-morpholino)propanesulfonic acid, pH 5.5], and treated for 15 min at 35°C in an ultrasonic bath (model RK 103H, 35 kHz, 2x280 W per period, Bandelin electronic, Berlin, Germany). Then, 500 µl of cell suspension were mixed with 9.5 ml MOPS buffer (2 mM, pH 7.0), stained with 2 µl SYBR® Green I (Life Technologies, Carlsbad, CA, USA) and shaken for 10 min in the dark. The suspensions were then filtered onto black polycarbonate filters (Merck Millipore, Billerica, MA, USA). The filters were dried and embedded in droplet of DABCO [25 mg of 1,4-diazabicyclo [2.2.2] octane in 1 ml of PBS (phosphate buffered saline) buffer plus 9 ml of glycerol] as antifading agent. Total cell counts were determined in an Imager M2 epifluorescence microscope (Zeiss, Steinheim, GER) equipped with the mercury lamp HXP 120 C and filter set 38 (GFP BP470) at a magnification of 1000x. 20 fields were counted for each sample.

3.4.4 Exoenzyme activity

The activities and kinetic parameters of the four major hydrolytic exoenzymes β-1,4-glucosidase (EC 3.2.1.21), β-1,4-xylosidase (EC 3.2.1.37), phosphatase (phosphomonoesterase, EC 3.1.3) and leucine aminopeptidase (EC 3.4.11.1) were determined in the soil samples employing highly sensitive fluorescence assays. The activities of the enzymes β-glucosidase, β-xylosidase and phosphatase were quantified following the liberation of methylumbelliferone (MUF) from MUF-β-1,4-glucoside, MUF-β-1,4-xyloside and MUF-phosphate, respectively. The activity of aminopeptidase was analyzed using aminomethylcoumarine (AMC)-leucine-hydrochloride (all Sigma-Aldrich; Steinheim, Germany).

Aliquots of 50 mg of the soil samples were mixed with 5 ml autoclaved ddH_2O at room temperature and enzymatic reactions were started by the addition of the MUF- or AMC-substrate analogues at concentrations of 5, 10, 20, 50, 100, 200 and 500 µM. Incubations without substrate analogues served as controls for the autofluorescence of the samples. The autocleavage of the substrate analogues was examined in ddH_2O without adding soil aliquots. During incubation, the soil slurries were shaken at 22°C and 140 rpm. After three hours of incubation 1 ml of the supernatant was removed and centrifuged for 5 min at 10,000xg. NaOH (final concentration 40 mM) was added to samples containing MUF and the concentrations of the liberated MUF or AMC were determined fluorometrically (excitation at 360 nm, emission at 450 nm) in an Infinite® M200 96 well-plate reader (Tecan; Maennedorf, Switzerland).

Some of the soils investigated contained higher amounts of clay minerals or organic matter that are expected to adsorb the liberated MUF or AMC which would result in an underestimation of exoenzyme activities (Coolen & Overmann, 2000) and an overestimation of the apparent K_M value (Nannipieri *et al.*, 2011). Based on the procedure developed earlier (Coolen & Overmann, 2000), soil aliquots were incubated with known amounts of MUF or AMC and residual concentrations of free fluorophores were quantified to determine the equilibrium adsorption isotherms for each soil and fluorophore. This yielded the affinity coefficient K [in ml·(g dry weight)$^{-1}$] and the dimensionless exponent n of the Freundlich equation which were used to calculate the total amount of fluorophore liberated in the exoenzyme assays (Coolen & Overmann, 2000). Both parameters of the adsorptive properties of the soils were also used as independent variables in subsequent multivariate analyses (see below).

Finally, the linearized version of the Michaelis-Menten formula as established for the determination of kinetic enzyme parameters in the presence of natural substrates was employed to determine the maximum enzyme activity V_{max} and the affinity parameter $K_m + S_n$ (the sum of the half saturation constant plus the concentration of natural substrate) from the concentrations A_{hydrol} of liberated fluorophore, the total concentration of substrate analogue A and the incubation time t_{inc} according to

$$\frac{A \cdot t_{inc}}{A_{hydrol}} = \frac{K_m + S_n}{V_{\max}} + \frac{1}{V_{\max}} \cdot A$$

(Coolen & Overmann, 2000).

3.4.5 Multivariate statistical analysis

The multivariate Redundancy Analysis (RDA) was performed using the *rda()* function from the R package "vegan" (Oksanen *et al.*, 2013; Borcard *et al.*, 2011). RDA associates two or more data sets in the multivariate ordination process. It allows to extract structures of a data set that are related to structures in another data set, and hence to formally test statistical hypotheses about the significance of these relationships. Conceptually, RDA is a multivariate multiple linear regression followed by a Principal Component Analysis (Borcard *et al.*, 2011). All numerical enzyme activity and environmental parameter data were scaled to mean = 0 and standard deviation = 1 prior to the analysis. The enzyme activity data were taken as "community data" matrix and the environmental parameters were taken as explanatory constraining matrix (see *?rda* for the manual on this function). The *rda()* function was used in its formula interface [*RDAobject <- rda(X ~ var1 + var2)* with default parameters; where *RDAobject* is the result of the RDA analysis, *X* is the "community matrix" and *var1* and *var2* represent two exemplary explanatory variables from the constraining matrix] in order to include also the categorical Land Use factors into the mainly numerical parameters containing explanatory matrix. The R code *anova.cca(RDAobject, step = 1000)* was used for a global test to assess the significance of correlation of environmental parameters with the dependent enzyme activities. Setting *by = "terms", anova.cca(RDAobject, by = "terms", step = 1000),* will perform separate significance test for each term (constraining variable). From the *RDAobject* the ordination scores for the first two axes were extracted (using scaling = 2) for the enzyme values, the sampling sites values, and the biplot scores for the explanatory numerical and categorical environmental parameters (using weighted average scores). The extracted scores were used to build the ordination plots using the R package "ggplot2" (Wickham, 2009).

In order to analyse which environmental parameters affect which properties of individual exoenzymes, a multiple linear regression was performed using the R code *lm(X ~ var1 + var2, data = Y)*, where X contains the values of the individual enzyme) and Y is the data frame containing all numerical environmental parameters.

3.5. Results

3.5.1 Soil physicochemistry, bacterial cell numbers and land use

All soils were sampled about 2 weeks after the end of the rainy season. An additional sampling campaign was conducted at Mashare before the start of the rainy season. The majority of the 77 soils examined in the present study were classified as arenosols. Forty of the soils had a low organic carbon (0.19-1.26 weight %) and total nitrogen content (0.016-0.097 weight %), a low water retention capacity, cation exchange capacity, low nutrient content and low pH (Suppl. Table 1). Darker, loamy sands were found in the riparian zones of the Cusseque and of the Okavango at Mashare as well as in four instances at the Mutompo site. These soils reached organic carbon contents of 1.66 to 4.34 weight % and nitrogen contents of 0.122 to 0.218 weight %, exhibited increased pH values and water retention capacities, and nutrient contents which exceeded that of the more sandy soils by a factor of two to three (Suppl. Table 1). Histosols and gleyosols were found in the peatlands of the Cusseque and were characterized by exceptionally high values of organic carbon (22.0-29.0 weight %) and total nitrogen contents (1.39-1.66 weight %) which exceeded the values of the other investigated soils by a factor of 10 to 15. The molar ratio for total N:total P determined for the ten Mutompo soils was 17.7 ± 4.0.

Comparable patterns were observed for the total bacterial cell numbers (TCN) in the different soils at the end of the rainy season. Nutrient poor sands and agriculturally used soils contained the lowest numbers of bacteria of $(0.2\text{-}0.6)*10^9$ cells $(g_{DW})^{-1}$. In contrast, cell numbers in the nutrient richer riparian woodland, bushveld savannah and pristine darker loamy soils of Mashare and Mutompo ranged between 1.2 and $4.3* 10^9$ cells $(g_{DW})^{-1}$ whereas TCN values in the Cusseque peatland soils C7 and C11 were another order of magnitude higher and reached $(15.3\text{-}21.0)*10^9$ cells $(g_{DW})^{-1}$ (Table 1). In order to assess the effects of drought, an additional sampling of Mashare soils was conducted in October 2011 before the rainy season 2011/2012. While the TCN values of the woodlands and dry fields darker loamy soils stayed rather constant or decreased, cell numbers in most other soils increased through the rainy season (Suppl. Table 1).

The three land use categories bushveld, dry agriculture and fallow were realized at all three study sites. In addition, two small scale pristine woodlands and several irrigated agricultural fields were available at Mashare. Sampling sites at Cusseque covered also 5 pristine woodland sites and one horticulture (Fig. 1).

3.5.2 Microbial exoenzyme activities and kinetics

The maximum hydrolysis rates V_{max} of β-glucosidase (BG), β-xylosidase (BX), phosphatase (PHO) and leucine aminopeptidase (LAP) varied largely between the 19 soils sampled at the Cusseque site. V_{max} values between 0.10 and 4.1 $\mu mol \cdot (g_{DW} \cdot h)^{-1}$ were determined for β-glucosidase, β-xylosidase, phosphatase in the nutrient poor sands and loamy sands whereas leucine aminopeptidase reached higher values of up to 16.3 $\mu mol \cdot (g_{DW} \cdot h)^{-1}$ in two of the soils (Fig. 2 A). Significantly higher activities of up to 122.8 $\mu mol \cdot (g_{DW} \cdot h)^{-1}$ were determined for leucine aminopeptidase in the two nutrient-rich peatlands. Values for β-glucosidase, β-xylosidase, phosphatase in the peatland horticulture soil C7 ranged between 7.3 and 15.3 $\mu mol \cdot (g_{DW} \cdot h)^{-1}$ and thus exceeded the respective exoenzyme activities in all other Cusseque soils.

In contrast to the high V_{max} values for leucine aminopeptidase observed for the Angolan soils, β-glucosidase displayed the highest activities in most of the soils studied in Northeast Namibia at Mashare (Fig. 2 B, C). Before the rainy season, the dry, nutrient-poor sandy soils of Mashare exhibited V_{max} values for all exoenzymes between 0.00 and 1.4 $\mu mol \cdot (g_{DW} \cdot h)^{-1}$, thus representing the lowest values that were determined in the present study (Fig. 2 B). Of all land use categories and soil types present at Mashare, pristine riparian woodlands and bushveld on loamy sands consistently reached the highest V_{max} values for β-glucosidase and leucine aminopeptidase before and after the rainy season [3.5 to 11.0 $\mu mol \cdot (g_{DW} \cdot h)^{-1}$] and surpassed the values determined for all types of agriculturally used fields on the same soil type. Only for some of the riparian woodlands and bushveld on loamy sand and only after the rainy season V_{max} values for β-xylosidase and phosphatase exceeded the respective values for the other soils. The rainy season exerted a stimulating effect on V_{max} in bushveld sands but not on sandy soils of other land use categories (Fig. 2 C).

Due the absence of perennial stream water, only three different types of land use were available at the southernmost Mutompo site (Fig. 1 D). Accordingly, ten different locations representing the different land use types were chosen for soil sampling. Highest activities of β-glucosidase, phosphatase and leucine aminopeptidase between 4.3 and 12.4 $\mu mol \cdot (g_{DW} \cdot h)^{-1}$ were detected at one site (M16) within the *Acacia* bushveld and high β-glucosidase activity was also found in a dry field (M14) that had only been established from bushveld only shortly before our sampling.

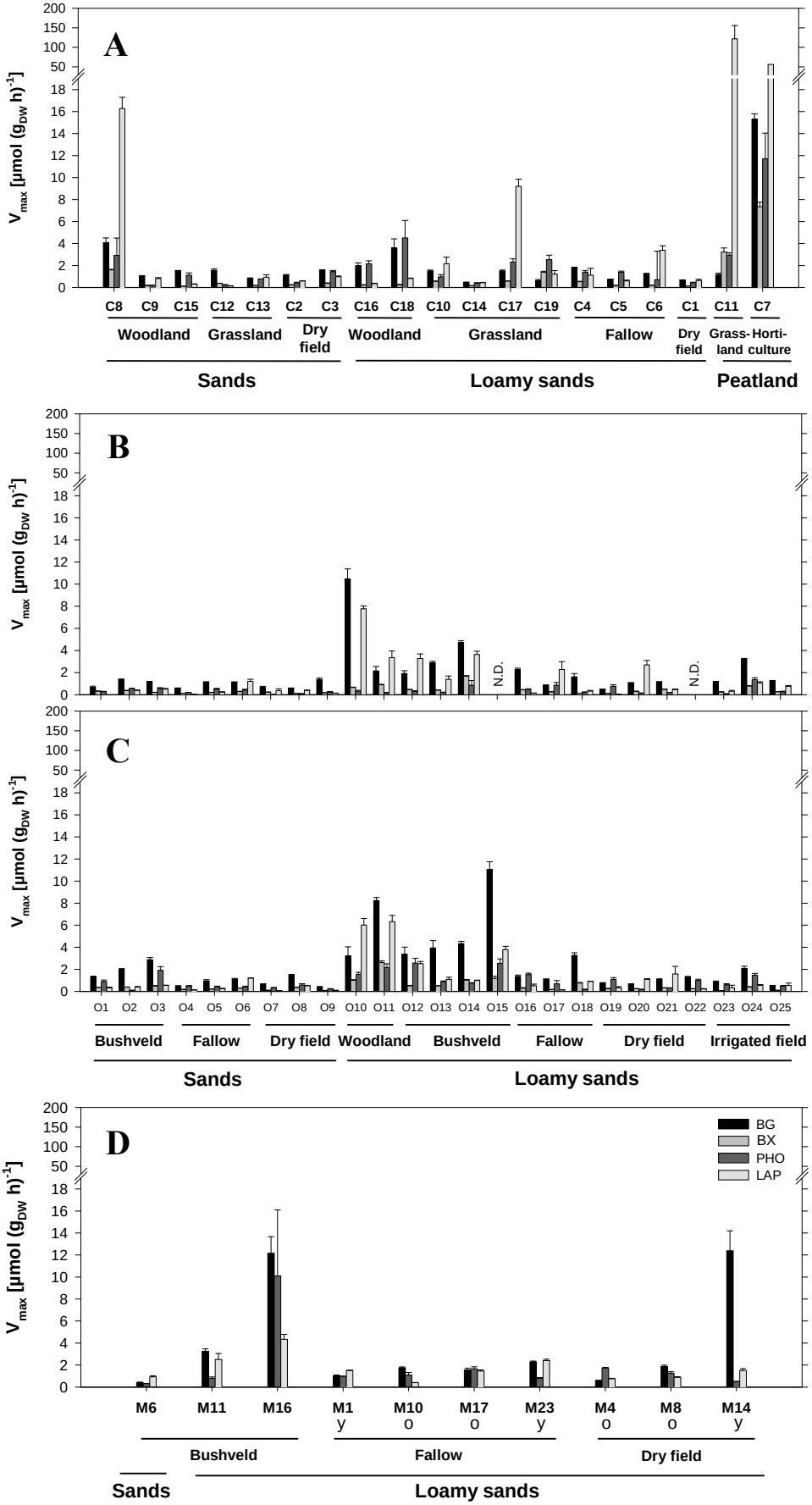

Figure 2: V_{max} values of the exoenzymes β-glucosidase (BG), β-xylosidase (BX), phosphatase (PHO) and leucine aminopeptidase (LAP) determined in 77 soils of the three study sites **A.** Cusseque (after the rainy season), **B.** Mashare (before the rainy season), **C.** Mashare (after the rainy season) and **D.** Mutompo (after the rainy season). Error bars represent standard error. BX activities could not be determined in Mutompo samples. Additional measurements for a fourth bushveld and a fourth dry field on loamy soils were conducted in March 2012 (panel **C**).

Since microbial exoenzymes are controlled by repression and induction, cell-specific exoenzyme activity can serve as an indicator of the availability of their respective substrates (Allison *et al.*, 2007; Geisseler & Horwath, 2009). In a parallel investigation (Huber *et al*. in prep.), high-throughput Illumina RNA sequencing of the microbial communities of woodland, bushveld and irrigated agriculture soils at Mashare revealed that active eukaryotes constituted only a minor fraction (1.0 to 20.9% of total ribosomal rRNA) whereas the active bacterial community prevailed in the soils (73.9 to 92.8% of total ribosomal rRNA) similar to semiarid Californian grassland soils that are dominated by bacterial biomass (Alster *et al*., 2013). Therefore the V_{max} data obtained in the present study were used to estimate specific activities of the four exoenzymes for bacteria. Since the cell volumina determined by microscopy showed only little variation (0.08 ± 0.031 μm^3) across all sampling sites, the cell-specific activities were based on the total bacterial cell numbers.

In Cusseque soils, specific β-glucosidase, β-xylosidase and phosphatase activities ranged between 0.06 and 9.7 $fmol \cdot (cell \cdot h)^{-1}$, but specific activities of leucine aminopeptidase were highly variable and reached up to 39 $fmol \cdot (cell \cdot h)^{-1}$ (Suppl. Figure S1 A). Differences in cell-specific activities of the four exoenzymes were less pronounced at the two other sampling areas (Suppl. Figure S1 B-D). The pronounced variability of specific LAP activities were analyzed further and were found to increase with V_{max} values until the latter reached about 10 $\mu mol \cdot (g_{DW} \cdot h)^{-1}$. Above this threshold, our data suggest a maximum of cell-specific rates that was reached in the peatland soils (C7 and C11) and woodland soil C8 (Fig. 3). The highly fertile horticulture plot C7 was planted with maize, tomatoes, potatoes, onions and sugar cane, and peatland plot C11 was located in densely grown grassland. Leucine aminopeptidase has been shown to be constitutively expressed in tomato and potato roots (Herbers *et al*., 1994; Tu *et al*., 2003). Theoretically, the increased activity could originate from the liberation of LAP during manipulation of plant roots which were more abundant in these soil samples than in sandy samples from the other sampling sites. However, an additional contribution of plant roots to LAP activities would yield a disproportionate increase of cell-specific LAP rates rather than the plateau observed for the values from C7 and C11. Instead, our data suggest that differences between soils exhibiting high V_{max} (C7, C8, C11) did not affect the specific LAP activity unlike in the remainder of the soils.

In most Angolan soils at Cusseque, $K_m + S_n$ values for β-glucosidase, β-xylosidase, phosphatase and leucine aminopeptidase ranged between 3.4 and 107 μM (Suppl. Fig. 2 A). However, higher $K_m + S_n$ values (145 to 466 μM) were detected for phosphatase in four of the

sands or loamy sands. Typically, the values for phosphatase or leucine aminopeptidase surpassed those of the other two exoenzymes.

Higher $K_m + S_n$ values were only observed before the beginning of the rainy season in one fallow and one dry field located in the old floodplains and in one fallow sandy soil at Mashare (535 µM for LAP) (Suppl. Fig. S2 B).

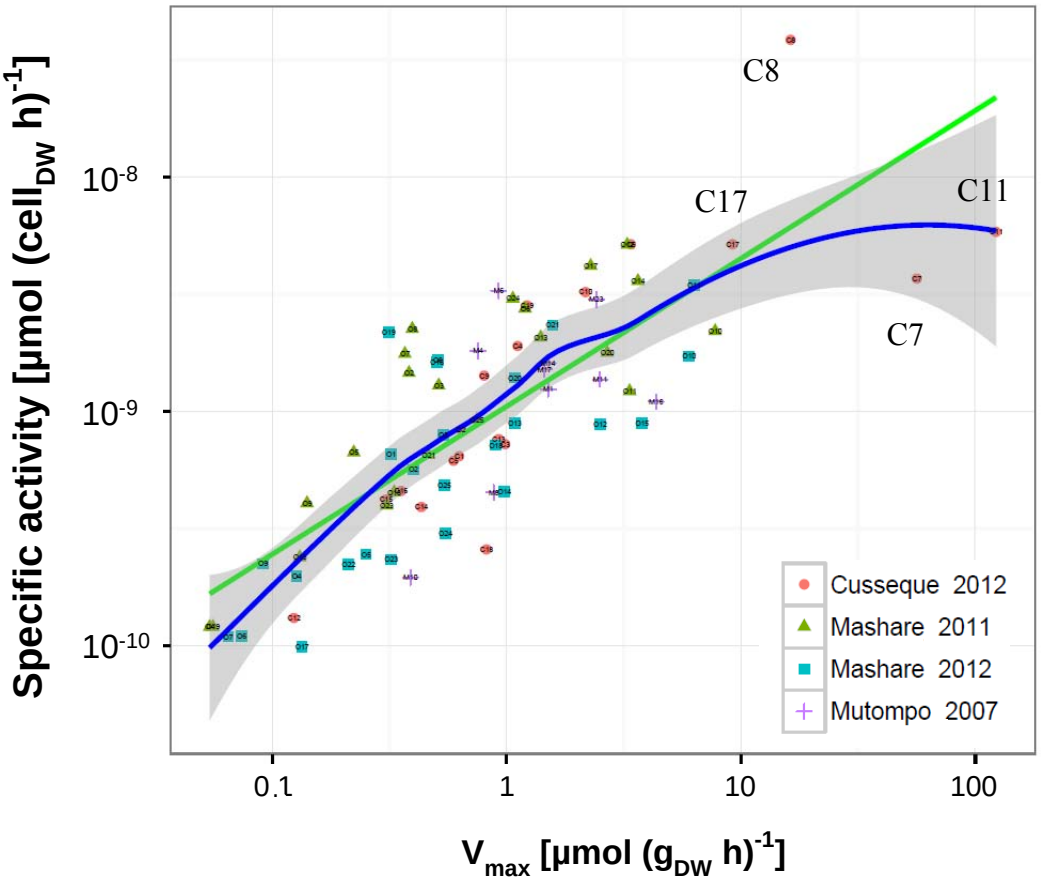

Figure 3: Interdependence between cell-specific leucine aminopeptidase activitiy and potential LAP activity V_{max} across the four sampling sites Cusseque (red circle), Mashare 2011 (green triangle), Mashare 2012 (blue square) and Mutompo 2007 (purple cross). A hyperbolic function (blue line) could be fitted to the data at high statistical significance, suggesting that cell-specific LAP rates reach saturation in the three soils (C8, C7, C11 at Cusseque) with highest LAP activities. The green line indicates the linear regression fit.

3.5.3 Multiple regression and multivariate statistical analysis

As a first step towards resolving the interrelation between exoenzyme activities, different environmental parameters and land use, a multiple regression analysis of individual exoenzyme activities was performed. Bacterial cell number was the only positive determinant for V_{max} values of PHO and LAP (Table 1). The Cl^-content was positively correlated with BG, while Cl^-, Mg^{2+} and PO_4^{3-} content was negatively correlated with LAP, BG and BX activities, respectively. Adsorptive properties of the soils affected BX positively and PHO negatively.

Table 1: Results of multiple regression analysis of the correlation between environmental parameters on V_{max} or cell-specific activity of the four exoenzymes. The *p* values are provided for positive (bold) and negative (italics) correlations.

Parameter	BG	BX	PHO	LAP
V_{max}				
TCN	-[1]	-	**0.0001**	**0.040**
Cl^-	**0.0007**	-	-	*0.042*
PO_4^{3-}	-	*0.001*	-	-
Mg^{2+}	*0.029*	-	-	-
K (AMC)	-	**0.039**	*0.020*	-
K (MUF)	-	**0.002**	-	-
Temp	**0.045**	-	-	-
Cell-specific activity				
TCN	*0.001*	*0.007*	*0.014*	*0.038*
Cl^-	**0.009**	-	-	-
HCO_3^-	**0.028**	-	-	-
NO_3^-	**0.037**	-	-	-
PO_4^{3-}	-	-	*0.042*	-
Mg^{2+}	*0.018*	-	-	-
n (MUF)	-	-	*0.012*	-
pH	-	-	-	**0.025**

[1] not significant at a level of $p < 0.05$

In order to elucidate potential drivers of the differential response of exoenzyme activities, a redundancy analysis was conducted for V_{max} of all four exoenzymes determined after the rainy season in Cusseque and Mashare (Fig. 4 A), and for the V_{max} values of β-glucosidase, phosphatase, aminopeptidase that were available for all 51 samples across the three geographic regions (Fig. 4 B). Multivariate analysis for the four exoenzymes yielded two principal components that together explained most (75.6%) of the variability by 12 of the environmental parameters (at $p < 0.05$) (Fig. 4 A). 67.7 % of the variation were explained by the two principal components for the activity patterns of β-glucosidase, phosphatase, aminopeptidase across all 77 soils (Fig. 4 B) and 6 environmental parameters were identified as significant environmental variables. The exoenzyme activities were positively correlated among each other (and hence found at an Eigenvalue of around 2 of RDA1). The analysis consistently identified total bacterial cell numbers as a major variable related to exoenzyme activity. In addition, organic carbon content, pH, and the concentration of some ions affected the exoenzyme activities. Along RDA 2, the C:N ratio, water content and adsorptive

properties [K(AMC)] of the soils were the main factors that exerted differential effects on exoenzyme activities (Fig. 4).

The strong correlation of V_{max} values with TCN that was consistently observed across all samples may suggest that potential hydrolytic activities in the arenosols are mostly determined by the number of bacteria rather than changes in cell-specific exoenzyme activities caused by different induction states of the cells. However, the results of a multiple regression analysis of cell-specific exoenzyme activities (Table 1) revealed a significant and positive effect of concentrations of three anions and a negative effect of Mg^{2+} on cell-specific β-glucosidase rates. Negative effects of phosphate concentrations and adsorptive properties of the soils were determined for phosphatase, and a positive effect of pH on cell-specific leucine aminopeptidase activity. Most notably, however, cell-specific activities of all four exoenzymes were negatively correlated with bacterial cell numbers. The respective RDA (Suppl. Fig. S3) showed that cell-specific activities of the exoenzymes correlated in a similar fashion to bacterial cell numbers and also adsorptive properties of the soils but that the cell-specific exoenzymes rates were affected in a differential manner by organic carbon and water content as well as the ion content of the soils.

Since specific LAP activities reached a maximum above a V_{max} value of 10 $\mu mol \cdot (g_{DW} \cdot h)^{-1}$ (compare Fig. 3), no effects of the conditions in soils exhibiting high V_{max} (C7, C8, C11) were apparent for the specific LAP activity. Therefore, the data points for the latter three soils were excluded from all of the above multivariate analyses. Parallel analysis of V_{max} and cell-specific activities by unconstrained principal component analysis yielded results (data not shown) comparable to those of RDA, indicating that major explanatory environmental parameters had been covered by the present study. No significant correlations of $K_m + S_n$ as dependent variable with any of the measured physicochemical parameters or land use were detected (RDA not shown).

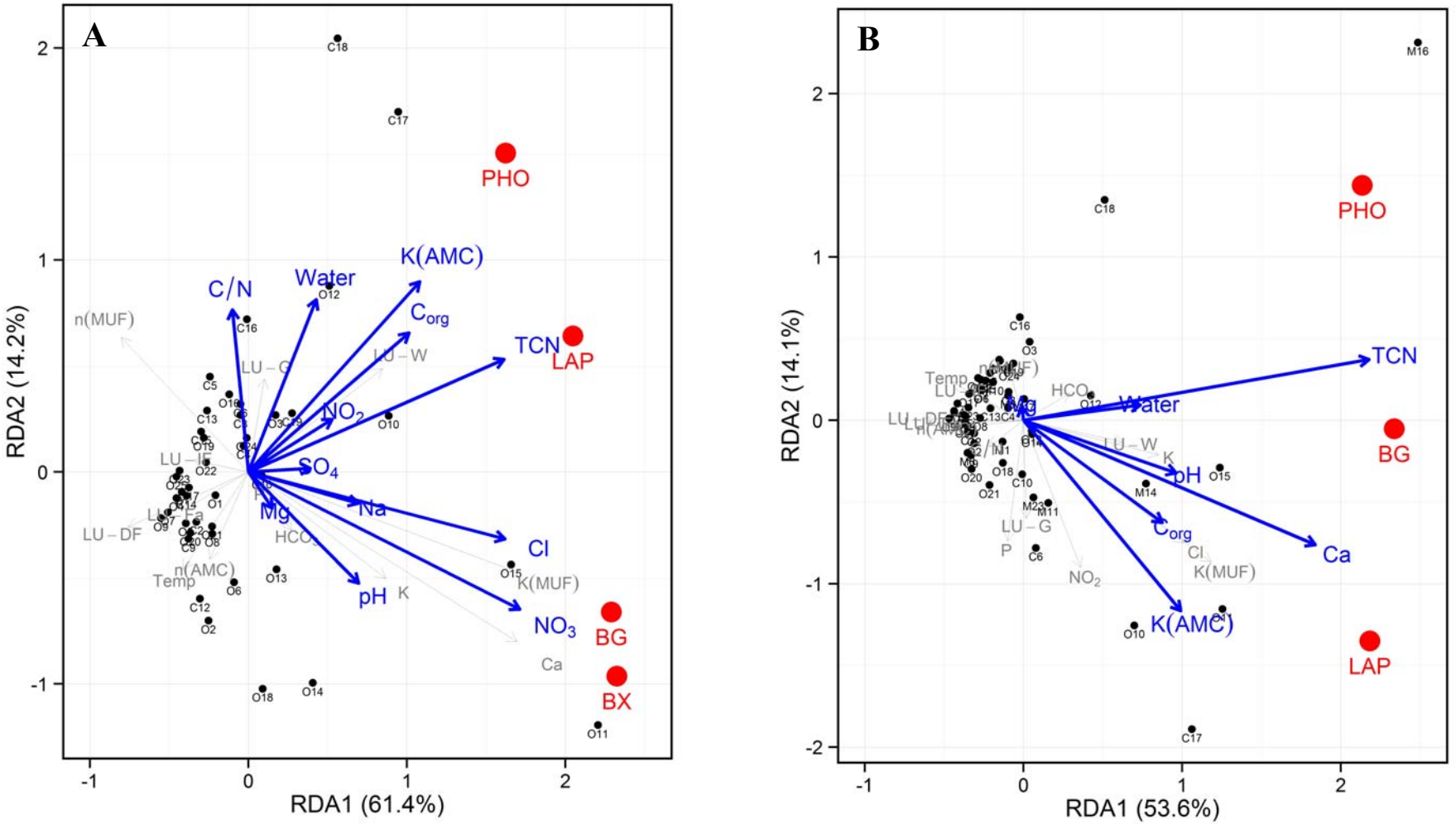

Figure 4: Redundancy analyses of the z-scaled V_{max} values obtained **A.** for the 4 exoenzymes at the Cusseque and Mashare sites after the rainy season (data for Mutompo omitted since no values for β-xylosidase were available) and **B.** for β-glucosidase, phosphatase, aminopeptidase determined at all three sampling sites Mutompo, Mashare and Cusseque after the rainy season. The exoenzymes are depicted as red circles and sampling sites as black dots. The ordination has been constrained using scaled numerical environmental parameters and land use as categorial factors. All environmental parameters are displayed as biplot arrows. Blue colour indicates those environmental parameters which contribute significantly (p-values in **A.**: pH, 0.001; TCN, 0.001; water content, 0.008; organic carbon content C_{org}, 0.001; C:N ratio, 0.001; Cl^-, 0.008; NO_3^-, 0.014; NO_2^-, 0.033; SO_4^{2-}, 0.001; Mg^{2+}, 0.03; Na^+, 0.017; adsorption parameter K(AMC), 0.015; in **B.**: pH, 0.001; TCN, 0.001; water content, 0.023; organic carbon content C_{org}, 0.009; Ca^{2+}, 0.011; Mg^{2+}, 0.029; adsorption parameter K(AMC), 0.03), grey colour indicates parameters with no signifcant contribution. The first two axis explain 75.6% and 67.7 % of the RDA variation in **A.** and **B.**, respectively.

3.6. Discussion

A comprehensive analysis of the interdependencies between soil hydrolytic activities and environmental parameters has only rarely been conducted so far. Most notably, data for low fertility soils of the semiarid Subsaharan Africa are largely missing. Our analysis provides novel insights into the controls and feedbacks of enzymatic processes in a soil type that prevails in areas of the Subsaharan central African plateau that are under increasing pressure by human land use.

3.6.1 Interrelation of geochemical soil conditions and bacterial activities in arenosols

Although direct evidence of P limitation of biological processes in tropical ecosystems remains scarce, phosphorus is assumed to represent the limiting nutrient in the humid tropics due to higher precipitation and longer geological periods of nutrient leaching than in the younger temperate and high latitude soils. So far, little information on nutrient cycling is available for the sandy savannahs in Subsaharan Africa. The molar N:P ratio in Mutompo soils was significantly higher (17.7 ± 4.0) than in other soils (mean 13.1± 0.8; Cleveland & Liptzin, 2007). On the opposite, the molar C:N ratios (14.6 ± 4.2) in the arenosols were very similar to those in soils from different latitudes (14.3 ± 0.5). The resulting average molar C:N:P ratio in arenosols amounts to 227:18:1 and differs from the worldwide average of 186:13:1 (Cleveland & Liptzin, 2007). Our chemical data therefore suggest that a pronounced phosphorus limitation of microorganisms also exists in Kalahari sands subject to the semiarid climate of northeastern Namibia and the temperate tropical climate with dry winters which prevail in central Angola.

Bacterial cell number was one of the strongest predictors of the V_{max} of the PHO and LAP and based on multivariate analysis exerted a similar influence on all 4 exoenzymes. This indicates that bacteria represent the main agents of carbon, nitrogen and phosphorus turnover in the Subsaharan arenosols and is in line with the low fraction of eukaryotic rRNA determined in a parallel investigation (Huber *et al.*, in prep.). Unlike plants, soil microorganisms maintain a rather constant molar C:N:P ratio (60:7:1; Cleveland & Liptzin, 2007) independent of the nutrient ratios in their environment and thus are expected to be particularly limited by phosphorus in the arenosols. Microbial exoenzyme activities provide insights into the effect of this nutrient limitation on the soil microbial community.

Our results for the V_{max} of β-glucosidase (≤ 15.3 $\mu mol \cdot (g_{DW} \cdot h)^{-1}$) are lower that those for a grassland cambisol (30-41 $\mu mol \cdot (g \cdot h)^{-1}$; Sanaullah *et al.*, 2011) but surpass values determined for Alaskan and Costa Rican soils (0.19 and 1.0 $\mu mol \cdot (g \cdot h)^{-1}$; German *et al.*, 2012). V_{max} values for β-xylosidase in the arenosols were in the same range as in a grassland

cambisol (3.6-4.4 μmol·(g·h)$^{-1}$; Sanaullah *et al.*, 2011) but higher than in a Haplic Phaeozem under different fertilization regimes (Kandeler *et al.*, 1999a). The V_{max} values for phosphatase reported for different soils range between 0.05 and 27.1 μmol·(g·h)$^{-1}$ but occasionally reach significantly higher values of up to 307 μmol·(g·h)$^{-1}$ (Allison *et al*. 2007; Nannipieri *et al.*, 2011). Values of up to 0.9 μmol·(g·h)$^{-1}$ and between 0.20-2.25 μmol·(g·h)$^{-1}$ have been detected in North American semiarid pinyon-juniper soil ecosystems (Krämer & Green, 2000) and under different fertilization regimes in a German Haplic Phaeocem (Kandeler *et al.*, 1999a), respectively. Thus the phosphatase values determined for arenosols (0.02-11.7 μmol·(g_{DW}·h)$^{-1}$) are comparable with most other soils in temperate latitudes. In some semiarid and arid soils, leucine aminopeptidase activities reached higher values than in most mesic soils (up to 316 μmol·(g·h)$^{-1}$; Sinsabaugh *et al.*, 2008). By comparison, only comparatively low values for leucine aminopeptidase activity (≤ 16.3 μmol·(g_{DW}·h)$^{-1}$) similar to those in mesic soils were found in the Subsaharan arenosols with the exception of the two peatland soils of Cusseque.

Across a wide variety of North American soils, activities of β-glucosidase and phosphatase, but not of leucine aminopeptidase, increased with soil organic matter content and the activities of all three enzymes positively correlated with soil pH (Sinsabaugh *et al.*, 2008). In different central European grassland and forest soils, the four exoenzymes which were also investigated in the present study were positively correlated with organic matter and total nitrogen content (Stursova & Baldrian, 2011). Soil temperature and moisture were good predictors for alkaline phosphatase activity in semiarid woodlands (Krämer & Green 2000) and soil organic matter was found to have a positive impact on phosphatase activity in drylands (Delgado-Baquerizo *et al.*, 2013). In contrast, none of the above environmental parameters did exert a significant effect across the series of arenosols investigated. Since the magnitude and variability of exoenzyme activities in Subsaharan arenosols were similar to other soils the former cannot be the reason for the absence of interdependencies with many of the environmental parameters.

In a global study of drylands, phosphatase activity, plant cover and organic matter content were identified as major determinants of total soil phosphorus (Delgado-Baquerizo *et al.*, 2013). The impact of phosphorus limitation on bacterial activity and the feedback on microbial nutrient cycling in low latitude soils have largely remained unexplored. The ratios between V_{max} values of BG:PHO (mean, 4.5; range, 0.25-32.7) determined in our study of Subsaharan arenosols clearly surpassed the mean value measured in North American soil sites across different climatic and geological regions (0.62; Sinsabaugh *et al.*, 2008), and our data

fall clearly outside of the correlation of the two enzyme activities seen in these other soils [1.41 ± 0.22 as opposed to a mean value of 0.959 and maximum of 1.1 for the ratio ln(BG):ln(PHO) in Sinsabaugh *et al*., 2008]. In fact, values similar to those from the literature (i.e. < 1.1) were only observed in the two peatlands (C7, C11) and one dark loamy grassland soil (C19) of Cusseque. The imbalance between carbohydrate and organic phosphate hydrolyzing enzymes suggests that the nutrient acquisition strategy of microorganisms in Subsaharan arenosols differs from that in the previously studied soil environments. This is further supported by the results of multivariate analysis that indicated that cell-specific activities of the four hydrolytic exoenzymes are controlled in a different manner by organic carbon, water content and the concentration of certain anions.

3.6.2 Specific bacterial responses to environmental conditions in arenosols

The $K_m + S_n$ values determined for β-glucosidase were in the same range as in other soils (Marx *et al.*, 2001; German *et al.*, 2012) and bacterial communities in freshwater (Chrost 1989). Similarly, $K_m + S_n$ values for leucine aminopeptidase were comparable to those in marine samples (Obayashi & Suzuki, 2005), whereas the values of phosphatase were higher than those in oligotrophic marine waters (Duhamel *et al.*, 2011). Based on our comparative assessment, bacteria in the African arenosols do not express exoenzymes with an unusually high affinity.

Given the dominance of bacteria in the arenosols, cell-specific exoenzyme rates also serve as proxies for the physiological state of the soil bacteria in these ecosystems. Hydrolytic enzymes are involved in the degradation of organic matter with rapid turnover times (Paul, 2007) and are induced by their respective substrates (Geisseler & Horwath, 2009). Interestingly, the specific activities of all four exoenzymes were inversely related to bacterial density in the arenosols, suggesting that bacterial exoenzymatic activity at high cell densities is not fully induced which for example could be related to end product repression.

In dryland soils organic phosphomonoesters constitute a readily available source of orthophosphate since up to 87% of the organic phosphorus compounds present are potentially hydrolyzable by phosphatases (Belnap, 2011). In temperate soils the cell-specific phosphomonoesterase activity is inversely correlated with soil P (Allison *et al*., 2007), since inorganic phosphate inhibits the expression of the *pho* genes (Van Dien & Keasling, 1998; Nannipieri, 2011). A similar mechanism of end product inhibition seems to operate in the arenosols where multiple regression analysis revealed a significant negative correlation between the cell-specific phosphatase rate and soluble phosphate. On the opposite, the

specific β-glucosidase activity did not decrease with increasing organic C contents as in other soils (Allison *et al*., 2007) and therefore is unlikely regulated by its cleavage product.

The saturation of cell-specific activities as well as strongly increased $K_m + S_n$ values for leucine aminopeptidase in Cusseque peatland soils support the view of substrate saturation in this particular environment independent of the actual land use (horticulture or grassland). The nitrogen contents determined in these two peatland soils exceeded the total nitrogen contents of the Namibian soils by one order of magnitude and might be the explanation for the very high aminopeptidase activities.

3.6.3 Implications for land management

Hydrolytic exoenzymes of soil bacteria represent rapidly responding indicators of soil fertility and show high sensitivity towards changes in the environment (Bandick *et al.,* 1999). Our study for the first time revealed that phosphate limitation in the Subsaharan arenosols that was inferred from the low content in agricultural plants (Pröpper *et al*., 2010) actually affects the major nutrient regenerating processes that are catalyzed by bacteria.

Increasing P limitation results in an increase of the plant N:P ratio towards the equator (Reich & Oleksyn, 2004). Accordingly, a low relative P content was previously also determined for plants of the Mutompo region (Pröpper *et al*., 2010). The availability of carbon and nitrogen in soils is determined by the balance between biological processes such as photosynthesis, N_2-fixation and decomposition, whereas the concentration of available phosphorus is also controlled by geochemical processes like rock weathering. Because water availability tightly controls the biological activity in drylands, the intensity of biogeochemical cycling of C and N versus that of P is expected to differ between humid and dryland soils. Indeed, a global assessment suggests that increasing aridity causes decreasing concentrations of organic carbon, carbohydrate, total nitrogen, and amino acids but increases the concentrations of inorganic phosphorus in drylands (Delgado-Baquerizo *et al*., 2013). Our results from Subsaharan arenosols qualify the conclusions drawn from these observations on a global scale. In nutrient poor arenosols, it appears unlikely that an increased availability of phosphorus will accompany the climate change predicted for the south central African plateau.

3.7. Acknowledgement

Many thanks to Alexander Gröngröft, Jona Luther-Mosebach, Lars Landschreiber (Institute for Soil Science, University of Hamburg) for all their help during the field campaigns in Namibia and Angola and for providing values for different soil environmental parameters. We thank Anja Stieler, Michaela Blank and Alicia Geppert (all DSMZ) for their permanent assistance and support in weighing several thousands of soil samples and Eva Romann (diploma student, Ludwig-Maximilians-University, Munich) for counting the Mutompo samples. The Namibian and Angolan governments and the local farmers and land owners are gratefully acknowledged for providing the opportunity in collecting soil samples and doing field incubations. Soil in Namibia was sampled under the collection permits 1138/2007 (of 7 March 2007), 1635/2011 (of 12 April 2011), exported under permits Nos.23001 (23 March 2007), 26053 (10 November 2011) and 27282 (5 April 2012) and analyzed under MTAs of the National Botanical Research Institute issued for samples D1-D29 (13 March 2008), JO-2011/2-RT1 to RT23 and JO-2011/2-TCN1 to TCN23 (on 9 November 2011) as well as samples JO-2012-RT1 to RT23 and JO-2012-TCN1 to TCN23 (on 5 April 2012). Collection and export of samples from Angola was conducted under the permit of March 31, 2012 issued by the Ministerio de Ensino Superior e da Ciencia e Tecnologia (Prof. Dr. Guilherme Pereira, Huambo). We thank the German Federal Ministry of Science and Education BIOTA (Biodiversity Monitoring Transect Analysis in Africa) and of the TFO (The Future Okavango) projects (BIOLOG/BIOTA project 01LC0621C, and TFO project 01LL0912M) which supported this work with grants to Jörg Overmann.

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3.9. Supplementary Tables

Supplementary Table S1: Overview of the 77 soil samples of the three different main sampling sites Mutompo 2007 (M1-23), Cusseque 2012 (C1-19), Mashare 2011 and 2012 (O1-25) listed with the main soil characteristics [soil type, land use type, pH, soil temperature, total cell number (TCN), total carbon, nitrogen and phosphorus content].
* indicates significant difference of TCN values at a level of $p < 0.05$

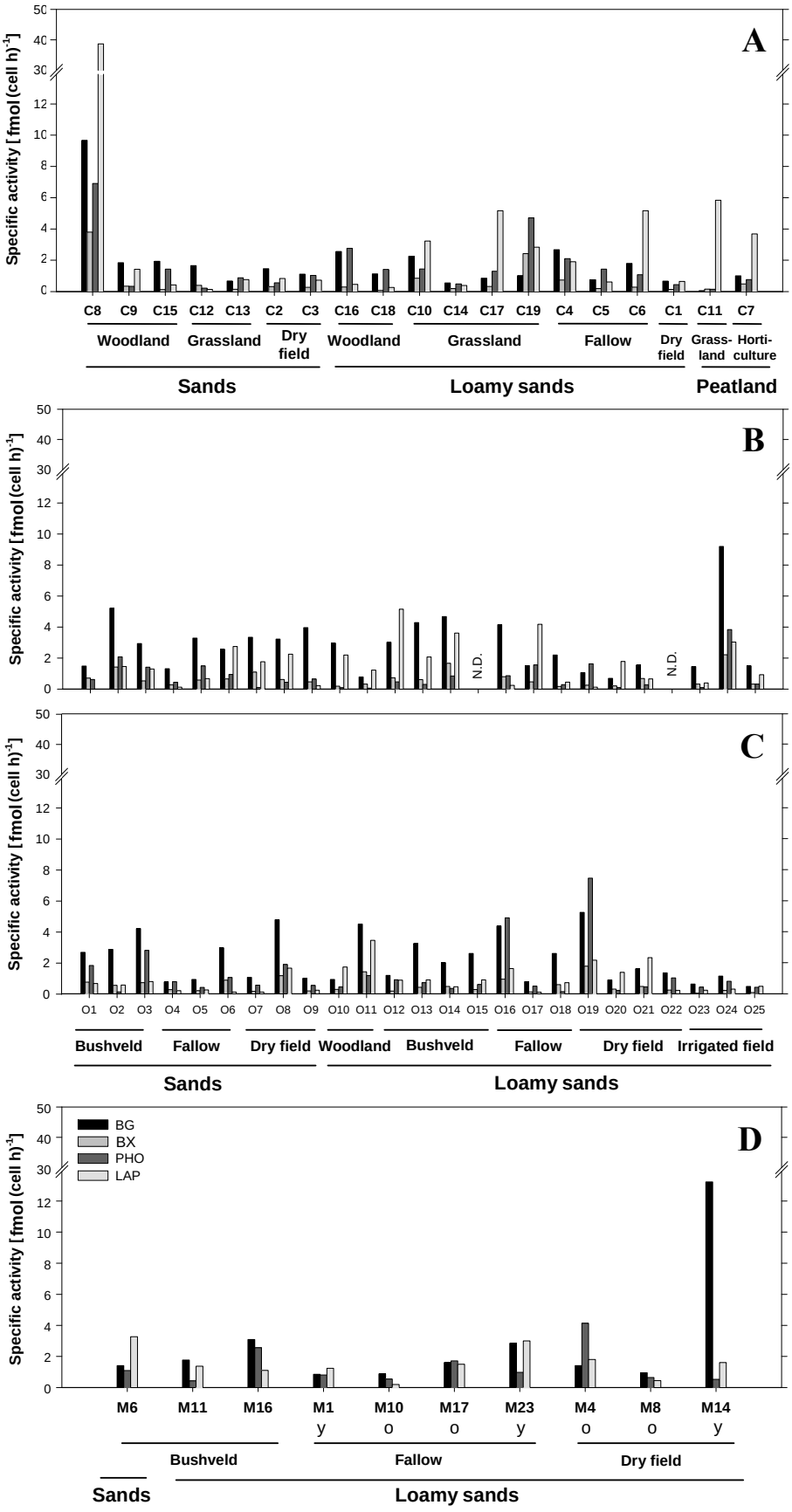
Sample	Latitude S	Longitude E	Elevation [m]	Soil/Land use type	Soil temperature [°C]	pH (H_2O)	TCN x 10^9 cells g^{-1} soil	C [weight %]	N [weight %]	P [g/kg]
M1	18°17´14.7´´	19°17´28.3´´	1167	LS/F	ND	7.2	1.21	0.42	0.040	0
M4	18°16´0.5´´	19°15´57.2´´	1163	LS/A	ND	6.6	0.42	0.31	0.034	0
M6	18°16´4.6´´	19°15´55.5´´	1163	S/BV	ND	5.7	0.28	0.57	0.042	0
M8	18°19´10.0´´	19°18´39.3´´	1168	LS/A	ND	7.2	1.96	0.48	0.045	0
M10	18°19´11.3´´	19°18´45.7´´	1168	LS/F	ND	7.2	2.00	0.42	0.037	0
M11	18°18´27.3´´	19°15´45.8´´	1167	LS/BV	27.9	6.6	1.83	0.45	0.047	0
M14	18°18´28,4´´	19°16´5.0´´	1167	LS/A	ND	7.1	0.94	0.39	0.038	0
M16	18°17´14.0´´	19°15´32.3´´	1165	LS/BV	ND	7.2	3.94	0.85	0.082	ND
M17	18°19´10.7´´	19°19´15.5´´	1170	LS/F	ND	6.6	0.96	0.55	0.048	0
M23	18°19´40.9´´	19°18´30.6´´	1168	LS/F	ND	7.2	0.80	0.29	0.028	0D
C1	13°42´44.39´´	17°3´51.19´´	1598	LS/A	32.2	6.1	0.98	0.85	0.057	0.007
C2	13°41´54.42´´	17°4´4.12´´	1593	S/A	33.2	6.1	0.77	0.79	0.049	0.008
C3	13°42´4.39´´	17°4´2.6´´	1546	S/A	31.9	6.3	1.36	0.88	0.065	0
C4	13°42´32.26´´	17°6´44.1´´	1551	LS/F	25.6	5.6	0.59	1.26	0.074	0.002
C5	13°42´43.31´´	17°3´55.76´´	1560	LS/F	28.6	5.4	0.97	1.07	0.064	0.003
C6	13°41´26.92´´	17°3´47.88´´	1563	LS/F	29.7	7.2	0.66	0.73	0.056	0.03
C7	13°42´8.03´´	17°4´34.97´´	1513	Pt/H	25.1	6.0	15.30	22.0	1.66	0.019
C8	13°41´16.84´´	17°4´48.79´´	1557	S/W	27.3	5.8	0.42	1.14	0.075	0.005
C9	13°41´22.6´´	17°3´57.85´´	1581	S/W	23,6	4.9	0.56	1.18	0.082	0.006
C10	13°41´17.2´´	17°5´16.37´´	1521	LS/G	28.4	6.1	0.67	0.70	0.053	0.002

C11	13°42′7.34′′	17°4′40.15′′	1514	Pt/G	21.5	6.1	21.00	28.95	1.39	ND
C12	13°41′16.55′′	17°6′22.32′′	1538	S/G	31.2	4.9	0.94	1.08	0.056	0
C13	13°41′16.33′′	17°6′6.32′′	1532	S/G	36.9	4.7	1.22	2.17	0.095	0.002
C14	13°41′16.19′′	17°5′31.02′′	1509	LS/G	36.1	4.8	1.11	0.97	0.055	0.003
C15	13°42′35.93′′	17°6′29.45′′	1542	S/W	23.7	5.3	0.73	1.73	0.083	0.009
C16	13°42′43.67"	17°3′53.21′′	1556	LS/G	25.6	5.9	0.78	1.25	0.074	0.003
C17	13°42′10.15′′	17°3′47.77′′	1525	LS/G	27.2	6.2	1.78	2.53	0.140	0.008
C18	13°41′58.52′′	17°4′8.15′′	1554	LS/W	26.2	6.4	3.19	0.79	0.061	0.002
C19	13°41′4.96′′	17°5′33.76′′	1509	LS/G	30.9	5.2	0.43	4.34	0.218	0.007
O1	17°54′51.91′′	20°12′28.44′′	1087	S/BV	30.7	6.7/6.2	0.44/0.49*	ND/0.49	ND/0.045	0
O2	17°54′41.26′′	20°10′42.02′′	1069	S/BV	34.8	6.0/6.7	0.26/0.71*	ND/0.39	ND/0.035	0
O3	17°53′58.85′′	20°12′34.31′′	1085	S/BV	26.9	6.6/6.8	0.40/0.68*	ND/0.39	ND/0.039	0
O4	17°54′21.92′′	20°8′47.98′′	1081	S/F	25.8	5.4/5.7	0.45/0.64*	ND/0.17	ND/0.018	0.008
O5	17°55′0.16′′	19°15′57.2′′	1084	S/F	25.3	6.6/6.7	0.33/1.03*	ND/0.27	ND/0.024	0
O6	17°55′4.94′′	20°9′6.16′′	1073	S/F	37.1	6.9/6.8	0.44/0.68*	ND/0.26	ND/0.036	0
O7	17°54′2.63′′	20°13′58.8′′	1079	S/A	25.4	5.5/5.7	0.21/0.60*	ND/0.19	ND/0.021	0.006
O8	17°54′9.25′′	20°14′4.52′′	1081	S/A	25.8	6.5/7.4	0.18/0.31*	ND/0.16	ND/0.016	0.01
O9	17°54′25.88′′	20°8′49.85′′	1084	S/A	23.8	5.6/5.7	0.35/0.41	ND/0.17	ND/0.019	0.004
O10	17°53′35.52′′	20°14′57.52′′	1061	LS/W	23.9	7.6/7.6	3.53/3.49	ND/1.98	ND/0.172	0.008
O11	17°52′37.99′′	20°15′20.84′′	1061	LS/W	24.8	7.1/6.8	2.74/1.83*	ND/1.66	ND/0.168	0.009
O12	17°53′34.87′′	20°10′59.2′′	1061	LS/BV	25.3	6.8/6.8	0.64/2.85*	ND/0.73	ND/0.073	0.005
O13	17°53′39.26′′	20°13′39.54′′	1051	LS/BV	26.8	7.0/6.6	0.67/1.22*	ND/0.54	ND/0.053	0.037
O14	17°53′32.86′′	20°12′25.49′′	1062	LS/BV	31.3	6.7/7.0	1.01/2.16*	ND/0.71	ND/0.063	0
O15	17°53′33.04′′	20°13′39.79′′	1060	LS/BV	26.1	ND/6.8	ND/4.25	ND/1.44	ND/0.122	0.005
O16	17°53′42.65′′	20°13′55.16′′	1062	LS/F	27.6	7.3/7.0	0.55/0.31*	ND/0.20	ND/0.021	0
O17	17°53′40.92′′	20°10′39.61′′	1068	LS/F	28.2	7.0/7.0	0.55/1.36*	ND/0.21	ND/0.021	0.004

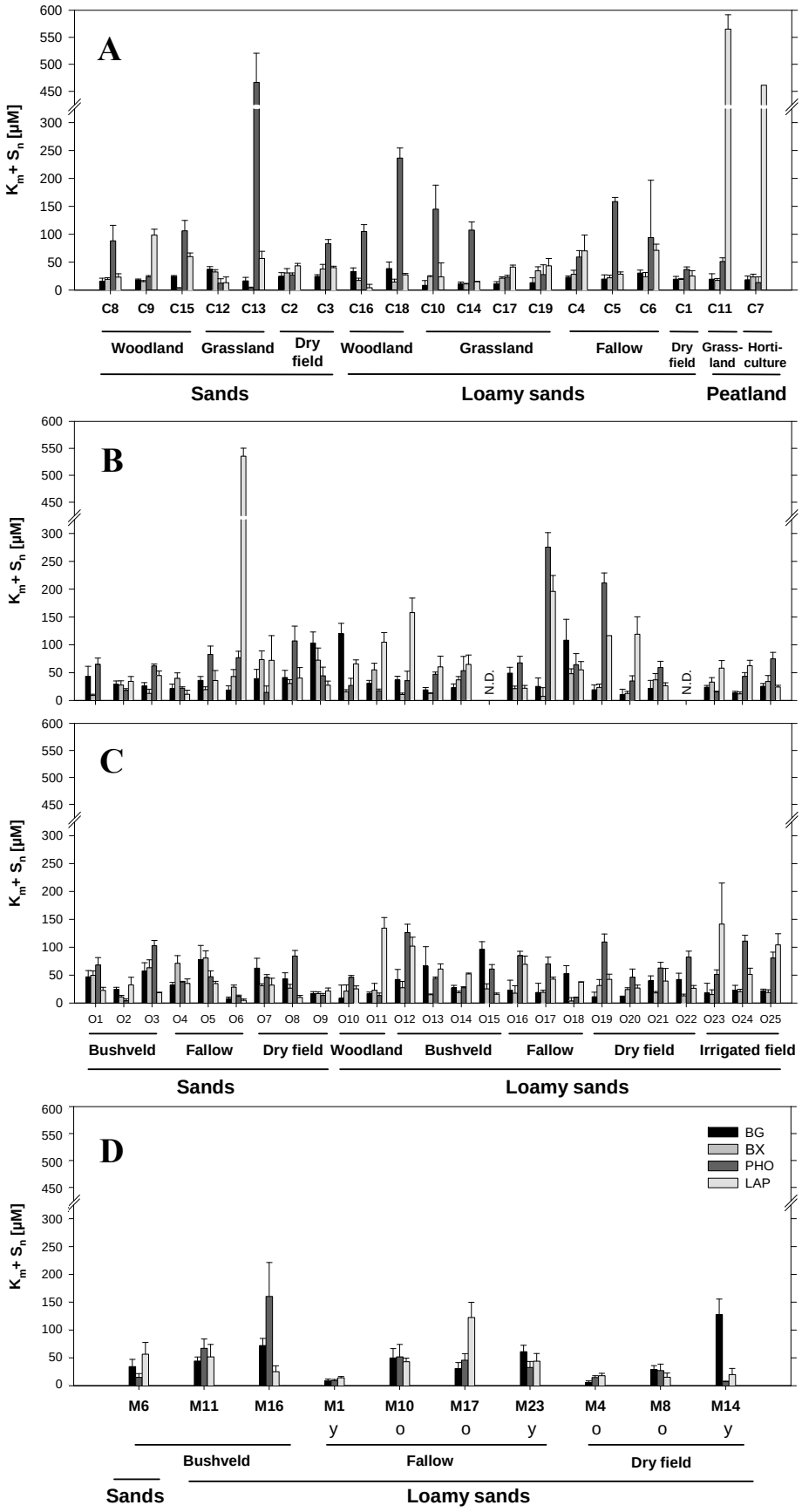
O18	17°53′37.93′′	20°14′50.71′′	1069	LS/F	30.9	7.9/7.7	0.74/1.26*	ND/0.51	ND/0.053	0.006
O19	17°53′43.66′′	20°13′59.34′′	1063	LS/A	27.4	7.0/7.2	0.46/0.15*	ND/0.24	ND/0.027	0
O20	17°53′48.84′′	20°9′7.88′′	1061	LS/A	26.7	8.3/8.2	1.51/0.78*	ND/0.34	ND/0.033	0.006
O21	17°53′31.78′′	20°10′16.5′′	1069	LS/A	28.0	8.2/8.3	0.71/0.68	ND/0.27	ND/0.027	0.013
O22	17°53′34.4′′	20°13′42.17′′	1060	LS/A	32.6	ND/6.7	ND/0.95	ND/0.28	ND/0.027	0.005
O23	17°53′32.14′′	20°10′57.79′′	1066	LS/IA	22.1	6.1/5.2	0.78/1.38*	ND/0.55	ND/0.045	0.017
O24	17°53′33.86′′	20°12′38.45′′	1062	LS/IA	26.3	7.6/7.1	0.35/1.82*	ND/0.48	ND/0.048	0.021
O25	17°53′32.39′′	20°11′15.4′′	1064	LS/IA	24.4	7.1/6.6	0.81/1.12*	ND/0.43	ND/0.043	0.021

Abbreviations: Soil type: S, sands; LS, loamy sands; Pt, peatland. Land use type: A, dry fields; BV, bushveld; F, fallow; G, anthropogenic grassland; H, horticulture; IA, irrigated fields; and W, woodland. ND, no data available.

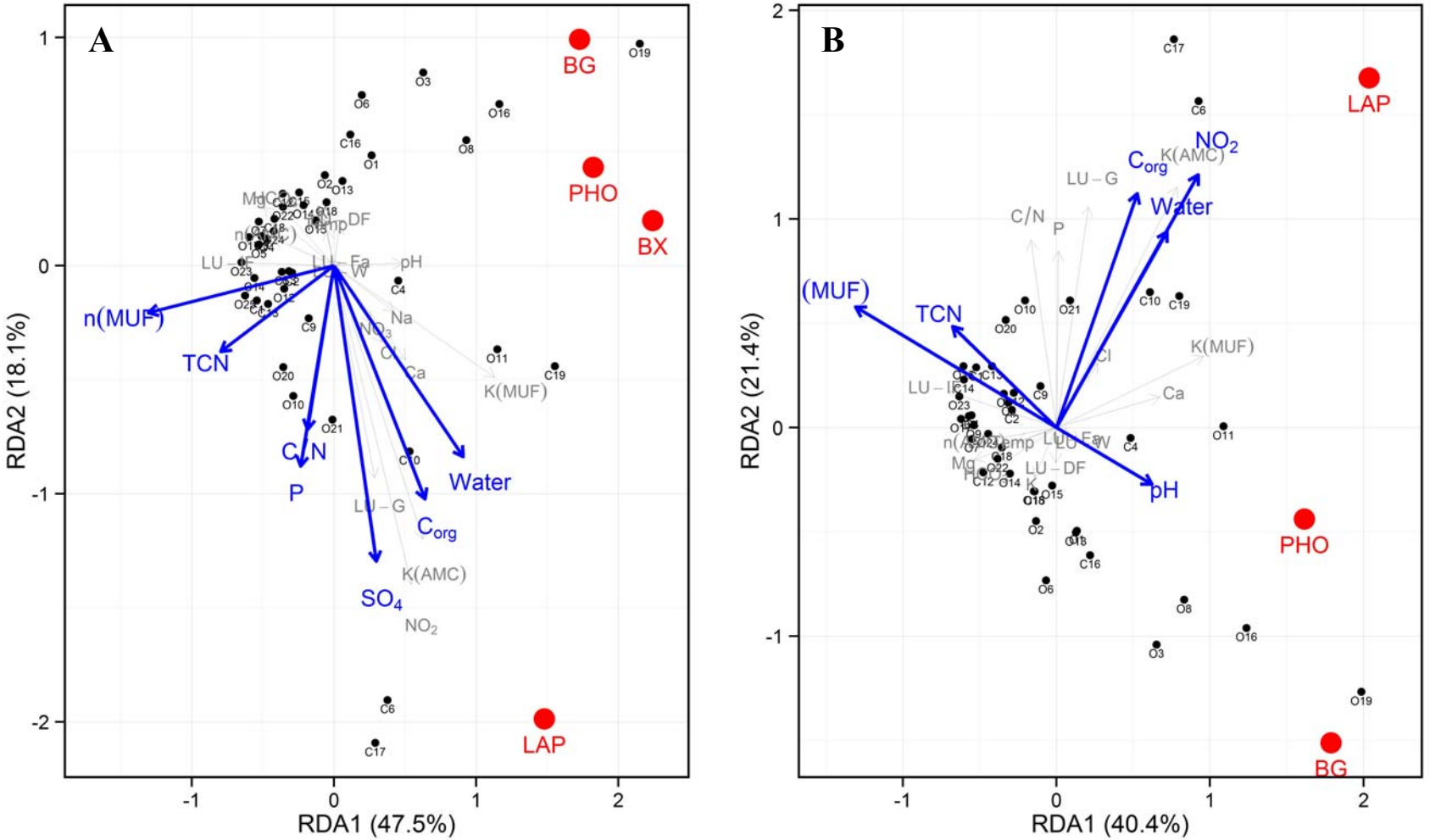
3.10. Supplementary Figures



Supplemetary Figure S1: Cell specific exoenzyme activities of β-glucosidase (BG), β-xylosidase (BX), phosphatase (PHO) and leucine aminopeptidase (LAP) determined in 77 soils of the three study sites **A.** Cusseque (after the rainy season), **B.** Mashare (before the rainy season), **C.** Mashare (after the rainy season), and **D.** Mutompo after the rainy season.



Supplementary Figure S2: $K_m + S_n$ values determined for β-glucosidase (BG), β-xylosidase (BX), phosphatase (PHO) and leucine aminopeptidase (LAP) determined in 77 soils of the three study sites **A.** Cusseque (after the rainy season), **B.** Mashare (before the rainy season), **C.** Mashare (after the rainy season), and **D.** Mutompo after the rainy season. Error bars represent standard error.



Supplementary Figure S3: Redundancy analyses of the z-scaled values of cell-specific exoenzyme activities obtained **A.** for the 4 exoenzymes at the Cusseque and Mashare sites after the rainy season (data for Mutompo omitted since no values for β-xylosidase were available) and **B.** for β-glucosidase, phosphatase, aminopeptidase determined at all three sampling sites Mutompo, Mashare and Cusseque after the rainy season. The specific exoenzyme activities are depicted as red circles and sampling sites as black dots. The ordination has been constrained using scaled numerical environmental parameters and land use as categorial factors. All environmental parameters are displayed as biplot arrows. Blue colour indicates those environmental parameters which contribute significantly (p-values in **A.**: TCN, 0.007; water content, 0.005; organic carbon content C_{org}, 0.033; C:N ratio, 0.02; P, 0.045; NO_2^-, 0.038; SO_4^{2-}, 0.037; adsorption parameter n(MUF), 0.029; in **B.**: pH, 0.041; TCN, 0.024; water content, 0.026; organic carbon content C_{org}, 0.036; P, 0.047; NO_2^-, 0.032; adsorption parameter n(MUF), 0.032), grey colour indicates parameters with no signifcant contribution. The first two axis explain 65.6% and 61.8 % of the RDA variation in **A.** and **B.**, respectively.

Chapter 4

Environmental controls and microbial drivers of nutrient cycling in low-fertility semiarid savannah soils

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4.1. Contribution of the authors

Katharina Huber collected all soil samples used for analysis in the Mashare core site and established several methods introduced in this study. Katharina Huber obtained the nitrogen turnover rates, i. e. the ammonification and nitrification, in nearly undisturbed soil cores and the exoenzyme activity measurements as well. The $^{15/14}N$ ratios of the filters were determined by Wolf-Rainer Abraham and Ulrich Struck. The total RNA preparation out of environmental soil samples was investigated by Katharina Huber. She also did the bioinformatic analysis of the 16S rRNA gene and therefore of the active soil microbial community after a bioinformatic introduction performed by Boyke Bunk, Pia Wüst and Sixing Huang. The figure and tables were composed by Katharina Huber with support of Johannes Sikorski and Pia Wüst in R questions concerning principal component analysis and heatmap calculation. Katharina Huber and Jörg Overmann wrote the present article together.

4.2. Abstract

A functional understanding of nutrient cycling in low fertility soils like Namibian Subsaharan savannah soils is an important basis for sustainable land use management. In order to identify considerable biogeochemical pathways and the according controlling factors, activities of four soil important exoenzymes and the ammonification and nitrification rates were investigated in Namibian soils differing in land use type. Additionally, the Pool Dilution Technique was obtained in the same soils at two different time points to determine the effect of water availability on nitrogen liberation processes as well. High input of degraded organic matter by exoenzymes (i. e. aminopeptidase) positively correlated with increased ammonification and nitrification rates in the riparian woodland and bushveld savannah soils. In contrast, lower input of degraded organic material as determined in agriculturally used soils implicated decreased nitrogen turnover rates. Drought effected nitrogen turnover rates and decreased ammonification and nitrification by a factor 2-4 in the riparian woodland and bushveld savannah soils. Nutrient stimulation experiments were conducted to identify key organisms of nutrient cycles. Illumina high-throughput total RNA sequencing of the stimulated soils revealed that both land use type and water variability effected the composition of the active soil microbial community. While *Firmicutes* dominated the irrigated fields, *Actinobacteria* and *Proteobacteria* prevailed in the riparian woodland and bushveld savannah soils. Drought decreased bacterial variability and abundances of the soil microbial community and supported the survival of microorganisms adapted to water stress like *Bacillus*, *Enterococcus* and *Arthrobacter*. Due to high abundance levels *Arthrobacter* and *Exiguobacterium* were identified as possible key players in the nutrient cycles of subtropical savannah soils.

4.3. Introduction

Semiarid and arid areas occupy about 40% of the continents (Fischer & Turner, 1978) and are expected to increase with climate change during the next years (Alley *et al.*, 2007). An estimated number of 100 million people live in these areas and almost exclusively depend on agriculture on low fertility soils (FAO, 2005). The sandy texture of the semiarid and arid soils, soil degradation, non-sustainable land use management and drought stress further decrease the inherently low fertility in these soils further (Greenland *et al.,* 2001; Nieder *et al.,* 2008; Parton *et al.,* 1987). Carbon, nitrogen and phosphorus are limiting nutrients in the Subsaharan Namibian savannah soils. Additionally, subtropical savannah soils in Africa show very low mineral fertilizer utilization rates, which ranged from 0.06 kg nutrients ha^{-1} per year in Congo Rep. to 53.2 kg ha^{-1} per year in South Africa in 2010 (mean value of sub-Saharan African countries: 10 kg nutrients ha^{-1} per year), in contrast to a mean mineral fertilizer utilization rate of 90 kg nutrients ha^{-1} per year worldwide (Greenland *et al.,* 2001), because the use of mineral fertilizers either is traditionally not established or too expensive in Subsaharan African countries. The sequential decline in soil fertility is explained by the loss of the macronutrients nitrogen or phosphorus, of micronutrients, by the decline of organic matter and biological activity and by the increase of toxicity as a result of enhanced release of particularly aluminum due to loss of soil organic matter as a buffer system (Marschner *et al.,* 2007; Nieder *et al.,* 2008; Greenland *et al.,* 2001). Consequently, the rapid loss of soil fertility and the decrease in crop yields promote shifting cultivation and increase conversion of pristine woodland and bushveld savannah to cultivated land. An alternative to current practice would be a sustainable land use management based on a functional understanding of the biogeochemical processes in semiarid and arid soils. So far, the interdependencies between nutrient turnover processes, their microbial drivers and environmental controls are largely unknown.

Major biogeochemical processes related to soil fertility such as nitrogen liberation (i. e. ammonification and nitrification) and the degradation of soil organic matter (SOM) are catalyzed by the soil microbial community (German *et al.*, 2012; Marx *et al.*, 2001; Sinsabaugh *et al*., 2008). In turn, the composition of the active microbial community is influenced by several environmental factors like water stress, temperature, nutrient availability, the present vegetation, pH and soil texture (Chau *et al.*, 2011; Fernández *et al.* 2011; Kuske *et al.,* 2011; Bartram *et al.,* 2014).

In order to obtain insights into the drivers of nutrient cycling in nutrient poor Savannah arenosols, the activities of soil relevant exoenzymes β-glucosidase, β-xylosidase, phosphatase

and aminopeptidase and nitrogen transformation rates were investigated in Namibian soils differing in land use type and water availability. Since exoenzymes degrade complex polymers they affect soil fertility by the liberation of carbon, nitrogen and phosphorus. Ammonification and nitrification provide ammonium and nitrate which can be assimilated by the soil community. As a third approach, the effects of different carbon, nitrogen or phosphorus compounds on microbial community composition were studied to identify soil microorganisms involved in nutrient cycling.

4.4. Materials and Methods

4.4.1 Study sites

In the present study the role of microorganisms in the nutrient cycling of different Namibian soils was examined as part of the BMBF (Bundesministerium für Bildung und Forschung, Germany) funded TFO (The Future Okavango) project. The soils chosen differed in land use type and sampling season and were collected in the basin of the Okavango river near the village of Mashare in the north-east of Namibia (17°53´40.92´´S, 20°10´39.61´´E; 1070 m above sea level; Fig. 1). The north-east of Namibia is characterized by a semiarid climate. Rainfall occurs during the summer months November to March and is followed by the dry season from April to October with an annual precipitation value of 595 mm m^{-1} and an annual mean temperature value of 22.3°C (AQUASTAT, 2014). The sampling campaigns were conducted in March 2012 after a normal rainy season and in March 2013 after a prolonged dry season. As a result soils varied in their soil water content.

The sampled soils belong to the FAO category of the arenosols and mainly consist of nutrient poor sands with a small percentage of loam due to former flooding by the Okavango river in the old flood plains. While arenosols are generally characterized by low soil organic matter content, low nutrient and water availability, low aggregate stability and low water

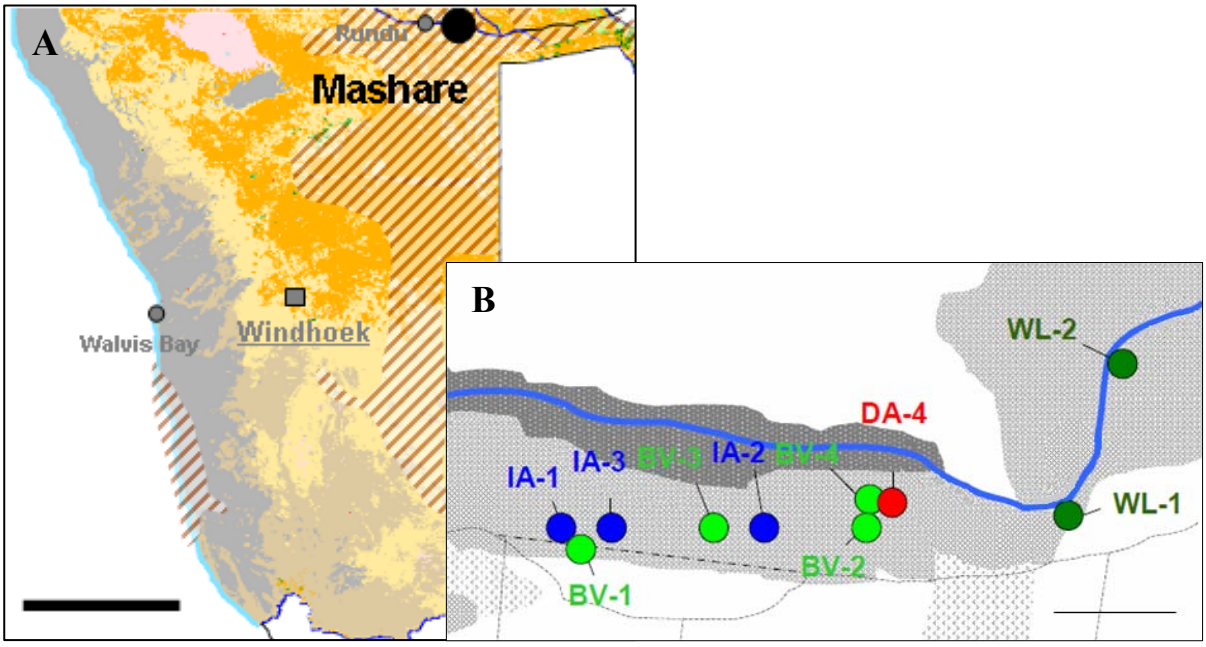


Figure 1: Location of the study site on the central African plateau. **A.** Overview map showing the Mashare sampling site. Green colors designate forested areas (dark green, evergreen; light green, open deciduous forest), orange and yellow colors shrublands and grasslands, respectively, and grey areas deserts. The hatched area indicates the geographic distribution of Kalahari sands (based on Hartemink and Huting, 2007).
B. Detailed map of Mashare sampling sites. Dark grey shading depicts wetlands of the Okavango river, grey shading old flood plains and light grey shading agriculturally used areas in the Kalahari sands. Dots indicate pristine woodlands (dark green), bushveld (light green), dry fields (red), irrigated fields (blue), and fallows (yellow). Dashed lines, roads.
Bars in **A.** indicate 300 km and in **B.**1 km.

retention capacity, the increased organic matter contents of soils in the old flood plains provide improved biogeochemical conditions.

The Mashare sampling sites represent the different land use types woodland (WL), bushveld (BV), dry fields (DA) and irrigated fields (IA). The agriculturally used fields around the Mashare village were dominated by *Sorghum bicolor*, *Pennisetum glaucum* (Mahangu), *Zea mays* and *Vigna unguiculata*. *Baikea plurijuga*, *Terminalia sericea, Combretum collinum* and different *Acacia* species like *Acacia nigrescens* prevailed in the pristine areas. The riparian woodlands were dominated by *Acacia nigrescens* and *Peltophorum africanum*, while the thornbush savannah was dominated by *Acacia erubescens and A. luederitzii* (Cauwer, 2013).

Characteristics of all sampling sites are listed in Tab. 1.

Table 1: Overview of the 10 soil samples of the two different sampling time points March 2012 and March 2013 listed with the main soil characteristics [land use type, pH, soil temperature, total cell number (TCN)]. Bold marked samples were used for the stimulation experiment and additional metatranscriptomic analysis.

Sample	Field campaign	Latitude S Longitude E	Soil temperature [°C]	pH (H_2O)	Water content [%]	TCN x 10^9 cell g^{-1} soil	C [weight %]	N [weight %]
OFP-WL-1	**2012/2013**	**17°53´35.52´´ 20°14´57.52´´**	**23.9/23.0**	**7.6**	**8.9/4.9**	**3.49/3.28**	**1.98**	**0.172**
OFP-WL-2	2012/2013	17°52´37.99´´ 20°15´20.84´´	24.8/23.2	7.1	4.7/1.4	1.83/0.69*	1.66	0.168
OFP-BV-1	2012/2013	17°53´34.87´´ 20°10´59.2´´	25.3/42.2	6.8	3.7/1.0	2.85/2.67	0.73	0.073
OFP-BV-2	**2012/2013**	**17°53´39.26´´ 20°13´39.54´´**	**26.8/33.1**	**7.0**	**4.5/1.1**	**1.22/0.77**	**0.54**	**0.053**
OFP-BV-3	2012/2013	17°53´32.86´´ 20°12´25.49´´	31.3/35.2	6.7	1.4/1.0	2.16/1.38	0.71	0.063
OFP-BV4	2012/2013	17°53´33.04´´ 20°13´39.79´´	26.1/27.6	6.8	4.3/1.1	4.25/0.95	1.44	0.122
OFP-DA4	2012/2013	17°53´34.4´´ 20°13´42.17´´	32.6/41.7	6.7	4.7/0.7	0.95/0.88	0.28	0.027
OFP-IA-1	2012/2013	17°53´32.14´´ 20°10´57.79´´	22.1/29.5	6.1	12.8/6.6	1.38/0.97	0.55	0.045
OFP-IA-2	2012/2013	17°53´33.86´´ 20°12´38.45´´	26.3/35.5	7.6	3.1/2.1	1.82/1.33	0.48	0.048
OFP-IA-3	**2012/2013**	**17°53´32.39´´ 20°11´15.4´´**	**24.4/30.5**	**7.1**	**3.9/3.3**	**1.12/0.88**	**0.43**	**0.043**

Abbreviations: OFP, old flood plains. Land use type: BV, bushveld; DA, drought agriculture; IA, irrigation agriculture; and WL, woodland.

4.4.2 Soil sampling

The soils of all three sampling campaigns were collected in 0-15 cm depth after removing the litter layer at the soil surface. Two intersecting sampling transects were marked in the field and four soil cores were collected at 5 m distance and along two perpendicular transects. The central intersection of the transects was sampled as well. This yielded a total of 9 samples per location which were pooled and homogenized. The pooled soil samples were kept at 4°C until activity measurements and sample preparation obtained in the laboratory.

Soil temperature was directly measured in the field in the top 10 cm of the soils (Checktemp 1 thermometer; Hanna Instruments, Kehl). The other physicochemical parameters were investigated using aliquots of the mixed soil samples. Dry weight was measured after drying 1 g of soil at 80°C for 72 h and pH values determined in distilled water as well as in 10 mM $CaCl_2$ solution.

4.4.3 Total bacterial cell numbers

For the determination of the total bacterial cell number (TCN) (Lunau *et al.,* 2005), samples were fixed directly in the field by mixing 100 mg of soil with 900 µl 1% glutaraldehyd fixation solution [v/v; 5.5 mM MES (2-(N-morpholino)ethanesulfonic acid as buffer, pH 6.0]. 50 µl of the resulting soil slurries were combined with 450 µl 100% methanol (v/v) and 1000 µl MES buffer (pH 5.5, 10 mM) and incubated for 15 min at 35°C in sonic bath (Sonorex Super RK 100 H, 35 kHz, 140 W). 500 µl of the suspension were stained with 9.5 ml of MOPS [3-(N-morpholino)propanesulfonic acid] buffer (2 mM, pH 7.0) and 2 µl SYBR Green I® Nucleic Acid Gel Stain (10,000x concentrate, Life Technologies, Carlsbad, CA, USA) for 10 min in the dark while shaking the samples. The mixture was filtered onto an Isopore™ polycarbonate membrane filter (0.2 µm pore size, 25 mm, GTBP02500, Merck Millipore, Billerica, MA, USA) by vacuum filtration. After drying, the filters were embedded in droplet of DABCO [25 mg of 1,4-diazabicyclo [2.2.2] octane in 1 ml of PBS (phosphate buffered saline) buffer plus 9 ml of glycerol] as an antifading solution. The TCN were determined by counting 20 major square fields with an average of 20-200 cells per sample at a Zeiss Imager M2 microscope equipped with a HXP 120 C mercury lamp, filter set 38 (GFP BP470) and at a magnification of 1000x (Zeiss, Oberkochen, Germany).

4.4.4 Exoenzyme activity assay

The activities and kinetic parameters of the four major hydrolytic exoenzymes β-1,4-glucosidase (EC 3.2.1.21), β-1,4-xylosidase (EC 3.2.1.37), phosphatase (phosphomonoesterase, EC 3.1.3) and leucine aminopeptidase (EC 3.4.11.1) were quantified

in the soil samples employing highly sensitive fluorescence assays based on the liberation of methylumbelliferone (MUF) from MUF-β-1,4-glucoside, MUF-β-1,4-xyloside and MUF-phosphate, respectively. The activity of aminopeptidase was analyzed using aminomethylcoumarine (AMC)-leucine-hydrochloride (all Sigma-Aldrich; Steinheim, Germany).

50 mg soil of the Mashare samples were incubated in 5 ml autoclaved ddH_2O at room temperature. The enzymatic reactions were started by the addition of the respective substrate analogue at a final concentration of 5, 10, 20, 50, 100, 200 or 500 µM. Soil aliquots incubated without substrate analogue served for the determination of the autofluorescence of the samples. The soil slurries were shaken at 22°C and 140 rpm for three hours. The autofluorescence of the soil samples was spectrometrically examined in parallel water samples without soil. After three hours of incubation 1 ml of the supernatant was removed and centrifuged for 5 min at 10000 x g. 2 M NaOH was added to the MUF assays to rise the pH. The amount of the liberated MUF or rather AMC was spectrometrically determined at extinction of 360 nm and emission of 450 nm in an Infinite® M200 96 well plate reader (Tecan, Maennedorf, CH).

Coolen *et al.* showed (Coolen & Overmann, 2000) that liberated fluorophores like MUF and AMC bind to soil particles, potentially leading to severe underestimation of exoenzyme activities. Therefore equilibrium adsorption isotherms were determined by incubating known amounts of MUF or rather AMC with the different soils and quantification of the concentrations of remaining free fluorophores. Exoenzyme activity values were calculated correcting for the adsorbed fluorophores employing the formula described in Coolen and Overmann (2000).

4.4.5 Nitrogen turnover rates (Pool Dilution Technique)

In March 2012 and 2013 the nitrogen turnover rates of ten Mashare soils differing in land use type (Tab. 1) were investigated directly in the field employing the Pool Dilution Technique modified after Barraclough (Barraclough, 1995). A plastic sheet was fixed on the soils to relocate incubated subsoil samples. For the determination of ammonification and nitrification rates, 666 µl of a 1.2 mM $^{15/14}NH_4Cl$ and $K^{15/14}NO_3$ salt solution (^{15}N containing salts: Sigma-Aldrich, St. Louis, MO, USA) with a 10% enrichment of the heavy nitrogen were injected with a needle (0.7 x 0.4 mm; B. Braun, Melsungen, Germany) throughout the top 4 cm of undisturbed soils. As a control, 666 µl of ddH_2O was injected in parallel soil sites. After 24 h soils were harvested with a sterile top-cut 10 ml syringe (Braun, Germany). Three soil subsamples from the same labeling spot were combined and treated as one parallel in the

following. Three parallels were run each of the ten soil sampling sites at the two different time points for both the ammonification and nitrification assays.

After collection 12 g (fresh weight) of soil were extracted in 50 ml 1 M KCl by shaking for 20 min. The soil extracts were filtered on low nitrogen filters (MN 614 1/4, Macherey-Nagel, Düren, Germany) and subsequently sterile filtered (Millex® GP syringe, pore size 0.22 µm; Merck Millipore, Darmstadt, Germany) to stop microbial activity. 30 ml of the filtrate were transferred to a 250 ml Erlenmeyer flask (Schott, Mainz, Germany) with a glass bead (0.8 cm in diameter). A glass fiber filter acidified with 20 µl 2.5 M $KHSO_4$ was inserted in the headspace of the flask by attaching it to the lid. Addition of 0.25 g heavy magnesium oxide to the filtrate transformed $^{14/15}NH_4^+$ to $^{14/15}NH_3$ which was trapped by the acidic glass fiber filter above. The acid GF-filter with the collected $^{14/15}NH_3$ was removed, exchanged by a fresh acidified filter and 0.25 g Devarda's alloy (mixture of Al, Cu and Zn) was added to convert the $^{14/15}NO_3$ in the filtrate to $^{14/15}NH_3$ which was trapped again by the acid GF-filter. After drying of filters in a speedvac (Thermo Fisher Scientific, Waltham, MA, USA) at 30°C, the $^{14/15}N$ ratio of the filters was analyzed in an IRMS (isotope ratio mass spectrometry; Finnigan™ MAT 252, Thermo Fisher Scientific). The ammonia on the filters is oxidized to nitrogen by combustion (1180°C) in the elemental analyzer. Afterwards, the gas is ionized by an ion source in the IRMS, separated in the magnetic field with respect to mass and ionization and subsequently analyzed in the detection system corresponding the molecular mass.

For the calculation of nitrogen turnover rates from isotope experiments, the soil dry weight and ammonium and nitrate concentrations need to be determined. Soil dry weight was determined by drying about 1 g of fresh soil for 3 d at 80°C. Ammonium and nitrate concentrations were measure employing colorimetric methods which were adapted to a 96 well plate format. For nitrate content measurements, 85 µl of the filtrate used for isotope dilution experiments were incubated with 4 µl of 5% (w/v) resorcinol and 110 µl concentrated sulfuric acid and spectrophotometrically determined at 360 nm (Alef & Nannipieri, 1995) in a 96 well plate reader (Infinite M200, Tecan, Maennedorf, Switzerland). In order to determine ammonium contents of the samples, 2 µl of the filtrate were incubated with 99 µl reagent A [3.4 % Na-salicylat, 2.5 % Na-citrat, 2.5 % Na-tartrat, 0.012 % Dinatriumpentacyano-nitrosylferrat, all (w/v)] for 15 min in the dark. Afterwards, 99 µl Na-hypochloride solution were added and the reactions incubated for 60 min in the dark. Finally, the ammonium contents were determined at 660 nm in a 96 well plate reader (Alef & Nannipieri, 1995).

The ammonification and nitrification rates were calculated following the formula introduced in Barraclough (1995).

4.4.6 Stimulation experiment and metatranscriptomics

4.4.6.1. Field incubation

Mixing and harvesting soils effect the soil microbial community in subtropical savannah soils since comparison of the results of CO_2 measurements under *in situ* and under *ex situ* conditions in a riparian woodland soil of Mashare showed that CO_2 efflux dramatically decreased by a factor of 4 due upon disturbances of the soil (data not shown). Therefore, the stimulation experiments were conducted directly in the field to keep disturbance effects (i. e. the transport, cooling and mixing effect) to a minimum and to study *in situ* conditions.

In 2012 and in 2013 a plastic sheet with 11 cm holes in diameter was fixed on the soil, which enabled the exact localization of the treated soils during the whole stimulation experiment (Fig. 2). Each spot area was supplemented with 10 ml of stimulation solution which were evenly spread over the soil at the beginning and after 2 and 4 days. The stimulants added in the five treatments comprised 10 mM KH_2PO_4 as an inorganic phosphorus source, 10 mM NH_4NO_3 as a nitrogen source, ARE (artificial root exudates; mixture of different sugars and carbon acids, Supplementary Tab. S2), 10 mM potassium phytate as an organic phosphorus and carbon source, and a mixture of ARE and 10 mM KH_2PO_4 as an inorganic phosphorus and carbon source. All nutrient solutions were adjusted to a pH of 7. Sterile ddH_2O was added as a negative control to assess the effect of water alone on the soil microbial community. Throughout the experiment, the soils were supplemented with 10 ml of sterile ddH_2O after 1, 3 and 5 days to alleviate a drying of the soils. After 6 days, 5 g of soil were harvested with a sterile top-cut 10 ml syringe (Braun) from each spot area and transferred to 10 ml LifeGuard™ Soil Preservation Solution (MoBio Laboratories, Carlsbad, CA, USA) and kept at 4°C during transport. Back in laboratory all 84 soil samples (Supplementary Tab. S1) were kept frozen at -80°C until the RNA extraction.

Figure 2: Stimulation experiment. *In situ* incubations under field conditions.

4.4.6.2. RNA extraction

For the preparation of the Illumina high-throughput sequencing the RNA was extracted from all 84 soil samples according the method described in (Lueders *et al.*, 2004) and modified by Bianca Pommerenke and Michael W. Friedrich (pers. comm.). All solutions were prepared with RNase free water and all surfaces were cleaned with RNase AWAY® (Roth, Karlsruhe, Germany).

0.6 g fresh weight soil, 0.7 g zirconium/silica beads (Ø = 0.1 mm; Roth), 750 µl 120 mM $NaPO_4$ buffer (pH 8.0) and 250 µl TNS-solution [pH 8.0; 500 mM Tris-HCl, 100 mM NaCl, 10% SDS (w/v)] were incubated for 45 s in the FastPrep®-24 Instrument (MP Biomedicals, Carlsbad, CA, USA) at 6.5 m s^{-1}. After the centrifugation for 20 min at 4°C and 20817 x g (Eppendorf 5417 R, Hamburg, Germany) the supernatant was transferred and mixed with one volume of phenol/chloroform/isoamylalcohol (25:24:1; pH 8.0) and centrifuged for 5 min at maximum speed and 4°C. The supernatant was mixed with one volume of chloroform/isoamylalcohol (24:1) and centrifuged for 5 min at maximum speed and 4°C. Afterwards the supernatant was transferred, mixed with two volumes of PEG solution [30% (w/v) polyethylene glycol 6000 in 1.6 M NaCl] and centrifuged for 90 min at 4°C and maximum speed for precipitation. The supernatant was removed and the pellet was washed by adding 500 µl ice cold 70% (v/v) EtOH and subsequent centrifugation for 30 min at 4°C. After removing the EtOH, the pellet was dried at room temperature and resuspended in 50 µl EB buffer (10 mM Tris-HCl, pH 8.5).

For the RNA Illumina high-throughput sequencing the DNA had to be completely removed from the RNA soil samples. First the DNA was digested with RNase free DNase I (1 U/ µl; Fermentas, Thermo Scientific) for 60 min at 37°C according to the instructions of the manufacturer. In order to stop the enzyme reaction, 1 µl 50 mM EDTA was added per 10 µl reaction mix and incubated for 10 min at 65°C. 1/10 volume 3 M Na-acetate (pH 5.2) and two volumes of isopropanol were added and the extract was incubated for 60 min on ice. After centrifugation for 5 min at room temperature, the liquid was removed. The pellet was washed by the addition of 500 µl ice cold 70 % (v/v) EtOH and subsequent centrifugation for 5 min at 20000 x g and room temperature. The final removement of the liquid was followed by the resuspendation of the pellet in 60 µl RNase free ddH_2O.

The remaining DNA fragments were removed using the RNeasy clean up kit (Qiagen, Venlo, Netherlands). After the addition of 150 µl 70 % EtOH (v/v) to the extract, the mixture was transferred to the RNeasy spin column and centrifuged for 15 s at 10400 rpm. After discarding the flow through, the membrane was washed by the addition of 700 µl RW1,

500 µl RPE buffer, and in turn 500 µl RPE buffer and centrifugation for 15 s, 15 s, and 2 min and removing the flow through, respectively. The column was dried by centrifugation at maximum speed for 1 min. The RNA was resuspended in 30 µl RNase free ddH_2O by centrifugation for 1 min at 10400 rpm. The pure RNA extracts were treated with 1 µl RNase-inhibitor (1U/µl; RiboLock RNase Inhibitor, Fermentas) and stored at -80°C.

4.4.6.3. Library preparation and Illumina sequencing

4.4.6.3.1. ScriptSeq Libraries

Illumina total RNA ScriptSeq Libraries without the depletion of rRNA were prepared by the sequencing service of the Helmholtz Center for Infection Biology (HZI, Braunschweig, Germany) using the ScriptSeq-v2-RNA-Library-Preparation-Kit (Epicentre, Illumina, MA, USA). The RNA concentrations ranged between 500 pg and 50 ng in total.

The reaction mix containing ddH_2O, RNA, fragmentation solution and cDNA synthesis primer was pipetted according to the manual instructions and incubated for 5 min at 85°C. Fragmentation of the RNA was terminated by cooling of the reaction to 4°C. For the following cDNA synthesis 4 µl of the cDNA master mix containing PreMix, 100 mM DTT and StarScript Reverse Transcriptase were mixed with the fragmentation reaction on ice. After the incubation for 5 min at 25°C and at 42°C for 20 min, the reaction was transferred to 37°C and 1 µl of Finishing Solution was added to the reactions. The reaction was proceeded for 10 min at 37°C and then for 3 min at 95°C. The mixture was cooled to 25°C to add 8 µl of the terminal tagging master mix containing DNA polymerase and terminal tagging premix to add the 3´terminal tag to the cDNA. The reactions were incubated for another 15 min at 25°C, stopped by incubation for 3 min at 95°C and finally cooled to 4°C.

The 3´terminal tagged cDNA was purified before amplification Agencourt AMPure XP system (Beckman Coulter, Pasadena, CA, USA). After warming of the beads to room temperature 400 µl 80% (v/v) EtOH and 45 µl of the beads were added to the samples. The reaction was incubated for 15 min at room temperature and then in a magnetic stand at room temperature for another 5 min until the liquid had cleared. The supernatant was discarded and the beads were washed by the addition of 200 µl 80% (v/v) EtOH. After incubation for at least 30 sec at room temperature, the liquid was removed without disturbing the beads. The washing step was repeated twice. After the last washing step the beads were air-dried for 15 min at room temperature. 24.5 µl ddH_2O were added, the tubes were removed from the magnetic stand and the reactions were mixed thoroughly. The reactions were incubated for 2 min at room temperature and put back to the magnetic stand for 5 min at room temperature until the supernatant with the scriptseq libraries appeared clear.

For the subsequent amplification, 22.5 µl of the purified reactions were mixed with 25 µl FailSafe PCR PreMix E, 1 µl forward primer, 1 µl barcode reverse PCR primer and 0.5 µl FailSafe PCR Enzyme (1.25 U). The amplification was performed by PCR with 15 repeating cycles of 95°C for 30 s, 55°C for 30 s and 68°C for 3 min and a terminal step of 68°C for 3 min. The PCR amplificate was purified according the method described above with 20 µl dissolving ddH_2O from the beads.

The quality of the ScriptSeq libraries was checked by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Barbara, CA, USA). Subsequently, whole metatranscriptome sequencing with 500 bp insert length was performed on a HiSeq 2500 Ultra-High-Throughput Sequencing System (Illumina, San Diego, CA, USA). 11 samples were run per lane. A total of 8.67-50.51 million reads per sample were generated during one 100 paired-end run.

4.4.6.3.2. Amplicons

Because of the high content of humic acids in the samples W02, W20 and W21 no proper scriptseq libraries could be prepared and produced. Additionally, sample IA05 yielded lower amounts of generated sequences compared to the mean of the other 80 samples. To gain information about the active microbial community even in soils with high humic acid contents, additional amplicon sequences were generated from these samples. The amplicon sequences were prepared following the modified method introduced by (Bartram *et al.*, 2011).

The first step in the preparation of the amplicon sequences was the cDNA synthesis using the GoScript™ Reverse Transcriptase System (Promega, Madison, WI, USA). 4 µl of 1:10 diluted RNA were mixed with 1 µl Random Hexamers (0.5 µg/µl), heated for 5 min at 70°C, centrifuged for 10 s and then cooled on ice. After the addition of 6.6 µl RNase free ddH_2O, 4 µl 5x GoScript Reaction Buffer, 2.4 µl 25 mM $MgCl_2$, 1 µl PCR Nucleotidmix (10 mM) and 1 µl GoScript Reverse Transcriptase (1 U/µl), the annealing step was performed for 5 min at 25°C and the extending step for 60 min at 42°C in a Veriti® 96-Well Thermal Cycler (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The incubation of the reactions at 70°C for 15 min inactivated the reverse transcriptase and stopped the cDNA synthesis.

The subsequent adaptation of the Bartram primers builds the basis for multiplexing several different samples in one ultra-high-throughput sequencing run/lane (Bartram *et al.*, 2011). The amplification of the V3 region of the 16S rRNA gene was also performed in the Veriti® Thermal Cycler employing the Phusion® High-Fidelity DNA Polymerase Kit (New England Biolabs, Ipswich, MA, USA). After mixing 16.6 µl PCR-ddH_2O, 10 µl 5x GC buffer Phusion, 1 µl 10 mM dNTP Mix Phusion, 1.5 µl 100% (v/v) DMSO, 0.2 µl forward primer

V3_F [50 pmol/µl; aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctCCTACGG GAGGCAGCAG; (Bartram *et al.*, 2011), 0.2 µl reverse primer V3_xR [50 pmol/µl; caagcagaagacggcatacgagatNNNNNNgtgactggagttcagacgtgtgctcttccgatctATTACCGCGGCTG CTGG; (Bartram *et al.*, 2011)], 0.5 µl Taq Polymerase Phusion (2 U/µl) and 20 µl cDNA, the reaction mix was incubated for 5 min at 94°C for an initial denaturation of the DNA. 15 cycles of denaturation (15 s at 94°C), elongation (15 s at 59°C) and annealing (15 s at 72°C) steps succeeded and the amplification was finished by a terminal annealing step for 7 min at 72°C followed by cooling of the reaction mix to 4°C.

Primer dimers were removed by gel purification using the MetaPhor® agarose (Lonza, Basel, Switzerland) which allows a very high resolution of the amplification products. The MetaPhor® agarose was mixed with 1x TAE (Tris, acetate, EDTA) buffer and incubated for 10 min in the fridge. After heating and preparing the gel, the gel polymerized for 30 min at room temperature and then in the fridge. The amplification product was completely filled in the gel which run for 4 h at 4°C and 80 mV. After the gel electrophoresis, the gel was stained in 0.01% SYBR Gold® Nucleic Acid Gel Stain (10000x concentrate in DMSO, Invitrogen, Life Technologies, Carlsbad, CA, USA) for 1 h. The bands were cut out and the DNA was extracted using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Merck, Darmstadt, Germany). The gel piece and 800 µl NTI buffer were incubated for 5 min at 50°C and transferred to the column. After centrifugation for 30 s at 11000x g the flow through was discarded and the column was washed by adding 700 µl NBT buffer and centrifugation at 11000x g. The washing step was repeated. In order to dry the column, it was centrifuged for 1 min at 11000x g and incubated for 5 min at 70°C. The DNA was dissolved by the incubation of the column for 1 min at room temperature with 12 µl TE-buffer followed by a centrifugation step. The elution step was repeated with another 12 µl TE-buffer for yielding 24 µl DNA in total. The pure DNA with the adapters for Illumina sequencing was delivered to the sequencing service GMAK of the HZI, Braunschweig.

4.4.6.4. Bioinformatic analysis/Metatranscriptome pipeline

The quality of the raw data for all 84 sample sequences was checked by the program FastQC version 0.10.1 (Simon Andrews; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). All raw reads from the 84 samples showed good per base sequence quality with mean Sanger quality values above 28 on the whole read length of the sequences with exception of W02. Despite repeated RNA extraction from the soil and repetition of ScriptSeq library preparation and a subsequent additional sequencing run, the per base sequence quality of W02 could not be improved. Therefore, W02 had to be omitted in further analysis. After the quality check,

the sequences were run through a metatranscriptome analysis pipeline, which had been established at the DSMZ with contributions of Boyke Bunk, Pia Wüst and Sixing Huang. The pipeline allows for processing of multimillions of metatranscriptome reads and consists of the following steps:

1. **Dimer Filtering**: A JAVA program called *DimerFilter*, which is based on the recognition algorithm of the FastQC program, purified the raw sequence data from potential primer dimers. Dimers, which might have been generated during amplification reaction were removed after the comparison of the sequences to a contaminant list containing the sequences of the Illumina adapters and the used PCR primers (Single End PCR Primer 1 (5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3' and the reverse sequencing primer without index 5'-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3'). *DimerFilter* also performs synchronization of the FASTQ-files from forward and reverse reads after filtering. However, the content of disturbing primer dimers in the raw sequences was rather low (0.1-4%).
2. **Chimera Check**: During amplification reaction the formation of chimeras occurred due to cross hybridization and mispriming events (Edgar *et al.*, 2011). To identify and filter generated chimeras, all remaining reads were subsequently checked with *Uchime* against the gold database provided by ChimeraSlayer, which is now integrated in *Usearch* 5.2.32 (Edgar *et al.*, 2011).
3. **Sorting of Sequences:** After the filtering of disturbing primer dimers and chimeras, the quality-checked remaining sequences were sorted into rRNA and mRNA fractions by the program sortMeRNA (Kopylova *et al.*, 2012). Using sortMeRNA, the generated sequences were compared against the SILVA and RFAM databases and the sequences sorted into the different RNAs (16S, 23S, 18S, 28S, 5.8S and 5S). The 16S rRNA fraction was passed to a taxonomic-dependent analysis (step 4). Sequences, which were not identified as rRNA sequences by sortMeRNA were regarded as mRNA sequences and passed to mRNA analysis.
4. **Taxonomic-dependent analysis of the rRNA fraction**: The identified 16S rRNA sequences were classified by the RPD classifier (Wang *et al.*, 2007; Cole *et al.*, 2009). The RPD classifier compared the gained sequences against the RDP database and assigns taxonomic ranks from domain to genus level due to their similarity to known and classified sequences. A confidence interval of 0.5 was applied justified by the short length (100 bp) of the sequences.

4.5. Results

4.5.1. Soil physicochemistry and total cell numbers

All soils were sampled two weeks after the end of the rainy season. However, soils sampled in 2013 had still been exposed to considerable drought due to the unusual low frequency of precipitation in the rainy season of 2012/2013. Despite affiliation with the FAO category of arenosols, three of the examined soils located in the riparian zone of the Okavango river (OFP-WL-1, OFP-WL-2 and OFP-BV-4 with a dense tree vegetation) exhibited increased carbon (1.44-1.98 weight %) and nitrogen contents (0.122-0.172 weight %). In contrast, lower carbon (0.278-0.729 weight %) and nitrogen (0.027-0.073 weight %) amounts were determined in the agriculturally used soils and in the other bushveld savannah soils which were used as meadow ground for cattle.

Similar patterns were examined for the total bacterial cell numbers. After the rainy season higher mean TCN values were reached in the riparian woodland and bushveld savannah soils than in agriculturally used soils and exceeded the values by a factor of three to four (Fig. 3 A). Moreover, the seasonal variations of water availability influenced the TCN values as well. All examined soils showed slightly decreased values after the prolonged drought of 2013 in comparison to the rainy season of 2012 (Fig. 3 A).

4.5.2. Exoenzyme activities

The activities of the four exoenzymes β-glucosidase (BG), β-xylosidase (BX), phosphatase (PHO) and aminopeptidase (LAP) were investigated in 10 Namibian soils which differed in land use type (woodland, bushveld, dry and irrigated fields). β-glucosidase displayed the highest activities in most of the soils studied in Mashare expect OFP-WL-1 reaching higher aminopeptidase activity values (Fig. 3 B). Of all land use categories present at Mashare, pristine riparian woodlands and bushveld savannah soils consistently reached the highest V_{max} values for β-glucosidase and leucine aminopeptidase after the rainy season in 2012 [3.2 to 11.0 $\mu mol \cdot (g_{DW} \cdot h)^{-1}$] and exceeded the values determined in agriculturally used soils by a factor of two to four (Fig. 3 B). Despite generally lower V_{max} values of the β-xylosidase, similar differences of riparian woodland and bushveld savannah soils compared to agriculturally used soils were determined (Fig. 3 B). However, one irrigated field achieved higher phosphatase activities than two bushveld savannah soils, but lower values than the other pristine soils (Fig. 3 B).

4.5.3. Nitrogen turnover rates: ammonification and nitrification in subtropical savannah soils

While the ammonification rates ranged from 1-24 µg N $(g_{DW}\, d)^{-1}$ in 2012 (Fig. 3 C) and from 2-14 µg N $(g_{DW}\, d)^{-1}$ in 2013 (Fig. 3 C), the nitrification rates ranged from 4-130 µg N $(g_{DW}\, d)^{-1}$ in 2012 (Fig. 3 D) and from 2-75 µg N $(g_{DW}\, d)^{-1}$ in 2013 (Fig. 3 D). Lowest nitrification values were determined in agriculturally used soils at both time points and in one bushveld soil after the prolonged dry season (Fig. 3 D). In contrast, both ammonification and nitrification reached highest turnover rates in the pristine woodland and bushveld savannah soils after the rainy season and at both time points, respectively (Fig. 3 C/D). Interestingly, one irrigated soil exhibited higher ammonification rates than the pristine soils after the prolonged drought. However, the values of this soil strongly varied within its parallels. Nitrogen turnover rates of the riparian woodlands and the bushveld savannah soils B1 and B4 exceeded the rates after the dry season by a factor of two to three (Fig. 3 C/D). Similar patterns were investigated with respect to the ammonification rates of the agriculturally used soils DA4 and IA3 (Fig. 3 C). In contrast, the ammonification values after the dry season determined in B3 and the other irrigated fields obviously exceeded the values after the rainy season (Fig. 3 C).

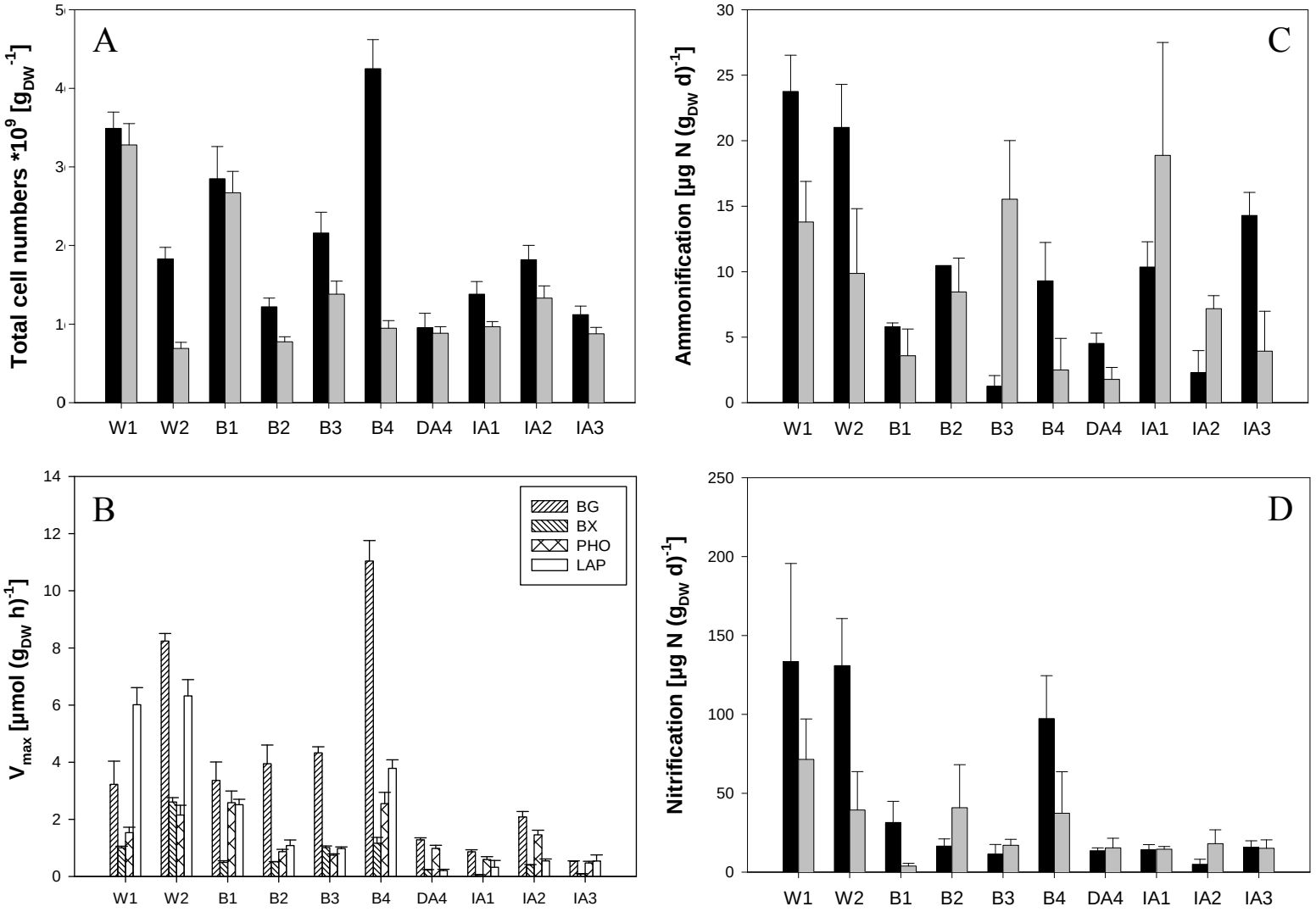


Figure 3: A. Total cell numbers, **B.** V_{max} values of the exoenzymes β-glucosidase (BG), β-xylosidase (BX), phosphatase (PHO) and aminopeptidase (LAP), **C.** ammonification and **D.** nitrification rates determined in 10 Mashare soils. The soils differed in the land use types bushveld (B), drought agriculture (DA), woodland (W) and irrigation agriculture (IA). Total cell numbers, ammonification and nitrification rates were determined in March 2012 (black bars) and March 2013 (grey bars). The soils differed in the land use types bushveld (B), drought agriculture (DA), woodland (W) and irrigated fields (IA).

4.5.4. Composition of the active soil microbial community

In March 2012 as well as in March 2013 the percentage of the 16S rRNA sequences of the total RNA sequences and therefore of the total active bacterial community ranged from 23-28% (Fig. 4). In contrast, the percentage of the 18S rRNA sequences to the total RNA sequences and hence of the active eukaryotic community reached obviously lower values (1-7%). Interestingly, the drought caused by the prolonged dry season of 2013 negatively effected both the active bacterial and the active eukaryotic community. But while the percentage of the active bacterial community decreased by only 10%, the active eukaryotic community was reduced by a factor of seven and four in the bushveld and woodland soils, respectively. Even in the irrigated fields the proportion of the active eukaryotes was decreased by a factor of two. Therefore, no obvious effect of the land use type on the abundances of active bacterial representatives was observed. However, a higher percentage of active eukaryotes was determined in the bushveld savannah soils than in the irrigated fields and woodland soils in 2012 (Fig. 4). Because of their high abundances in the active soil microbial community, the subsequent analysis focused on the bacteria.

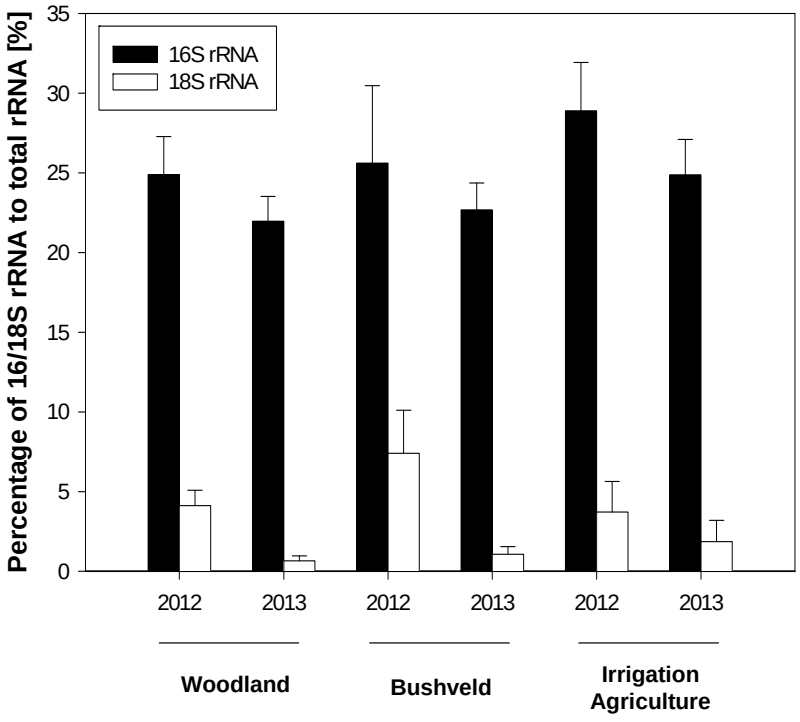


Figure 4: Percentage of mean 16S rRNA (black bars) and 18S rRNA (white bars) to total rRNA sequences, respectively. The sequences were determined in the 84 soil samples of the stimulation experiment differing in the land use types woodland, bushveld and irrigation agriculture and in the sampling time points 2012 and 2013.

To visualize the impact of environmental parameters and the added nutrients on the soil bacterial community, the abundances of the 16S rRNA Illumina sequences were plotted in a heatmap. A principle component analysis of the bacterial community composition with a posthoc analysis of the influence of season, land use type and nutrient stimulation was conducted (Fig. 5/6; Suppl. Fig. S1-5). 97.8% of the abundance variation of the soil community composition were explained by the parameters land use type, season and added nutrients. The *Proteobacteria, Firmicutes* and *Actinobacteria* were the most important bacterial phyla determined in the Illumina sequences of the stimulation experiment. Even within these three prevailing phyla *Proteobacteria, Actinobacteria, Firmicutes* and the soil important *Acidobacteria* 72.2%, 75.6%, 71.6% and 81.3% of the abundance variation on genus level were explained by the variations of the examined environmental parameters, respectively (Fig. 6). But the land use type and the sampling time point and therefore the water availability in the soils seemed to exert the most significant effect on bacterial abundance (Suppl. Fig. S1-5).

Rhizobiales as part of the *Proteobacteria* showed highest abundances in the riparian woodland and bushveld savannah soils (Fig. 6 C; Suppl. Fig. S3). Interestingly, after the prolonged dry season of 2012/2013 highest abundances of *Enterobacteriales* were determined in these soils and the activity patterns of the *Rhizobiales* were clearly decreased (Fig. 6 C). In irrigated soils high activity patterns of the *Pseudomonadales* were determined at both sampling time points (Suppl. Fig. S3). But not only the water availability and the land use type effected the activity of the *Proteobacteria*. The added nutrients influenced the clustering of the soils and therefore the bacterial activity patterns in the soils as well. Especially the addition of phosphorus containing nutrients clustered the respective soils within the three different land use types and the two different time points in close proximity (Fig. 6 C; Suppl. Fig. S3).

Within *Actinobacteria* the *Micrococcaceae* - especially the genera *Arthrobacter*, *Microbacterium* and *Curtobacterium* - were highly abundant in irrigated fields and in the riparian woodland soils in 2012 and 2013 (Suppl. Fig. S5). Additionally, increased activities of the *Streptomycetaceae* and of the *Propionobacteriaceae* were determined in some irrigated fields. The bushveld soils of both sampling campaigns showed slightly increased activity values of the *Nocardioidaceae, Geodermatophilaceae, Pseudonocardiaceae, Micromonosporaceae* and *Microbacteriaceae* (Fig. 6 B; Suppl. Fig. S5). Furthermore, high abundance levels of these *Actinobacteria* families affiliated with the addition of different phosphorus sources (Fig. 6 B; Suppl. Fig. S5).

While after the rainy season of 2011/2012 *Bacillus* and *Sporosarcina* were the most active *Firmicutes*, the *Enterococcaceae* represented the most active ones in the riparian woodland and bushveld savannah soils after the prolonged dry season of 2012/2013 (Fig. 6 D; Suppl. Fig. S4). However, the irrigated fields showed high abundance levels of *Exiguobacterium* in 2013. Increased activity levels of *Paenibacillus* were determined in the irrigated fields and in some representatives of the bushveld savannah soils (Suppl. Fig. S4). The activity of the *Firmicutes* seemed to be influenced by the phosphorus limitation in the Subsaharan savannah soils as well. Especially the soils supplemented with different phosphorus sources clustered together (Fig. 6 D; Suppl. Fig. S4).

Extraordinary high activity levels of subdivision 6 *Acidobacteria* in the riparian woodland soils were determined after the prolonged dry season of 2012/2013 (Fig. 6 A; Suppl. Fig. S2). In the riparian woodland soils sampled in March 2012 and in the bushveld savannah soils at both time points high abundance levels of subdivision 4 and 6 and low abundance levels of subdivision 3 were obtained (Fig. 6 A; Suppl. Fig. S2). However, in irrigated fields decreased abundances of subdivision 6 and 4 and increased abundances of subdivision 3 were determined as in the riparian woodland and bushveld savannah soils (Fig. 6 A; Suppl. Fig. S2). In the irrigated fields the addition of NH_4NO_3 resulted in a clustering of the samples collected in March 2012 and March 2013 (Fig. 6 A; Suppl. Fig. S2). Interestingly, subdivision 16 - also an unknown and uncharacterized subdivision until now, seemed to be stimulated by the addition of phosphorus sources in the irrigated soils (Fig. 6 A; Suppl. Fig. S2).

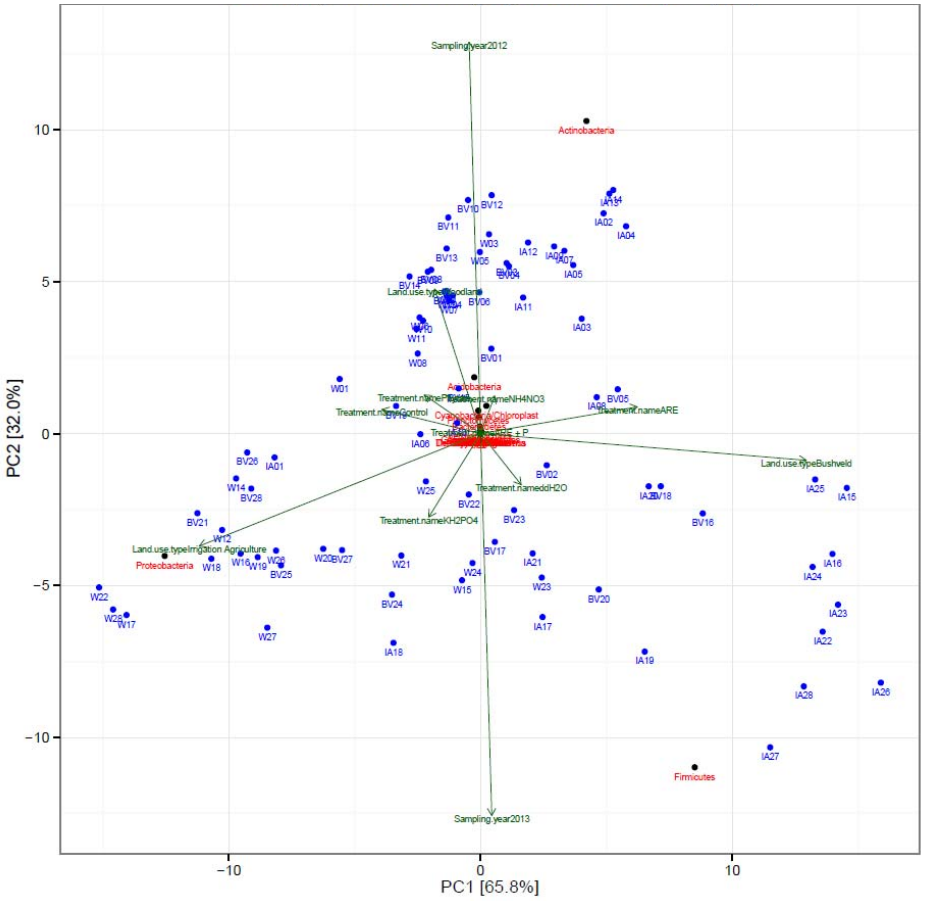
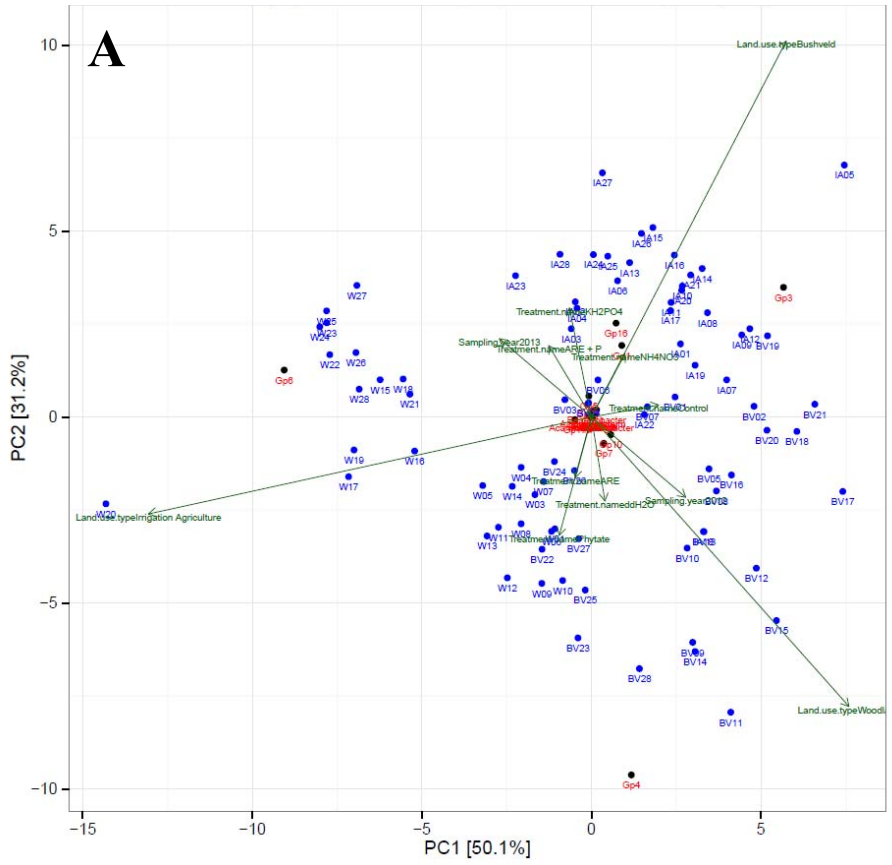
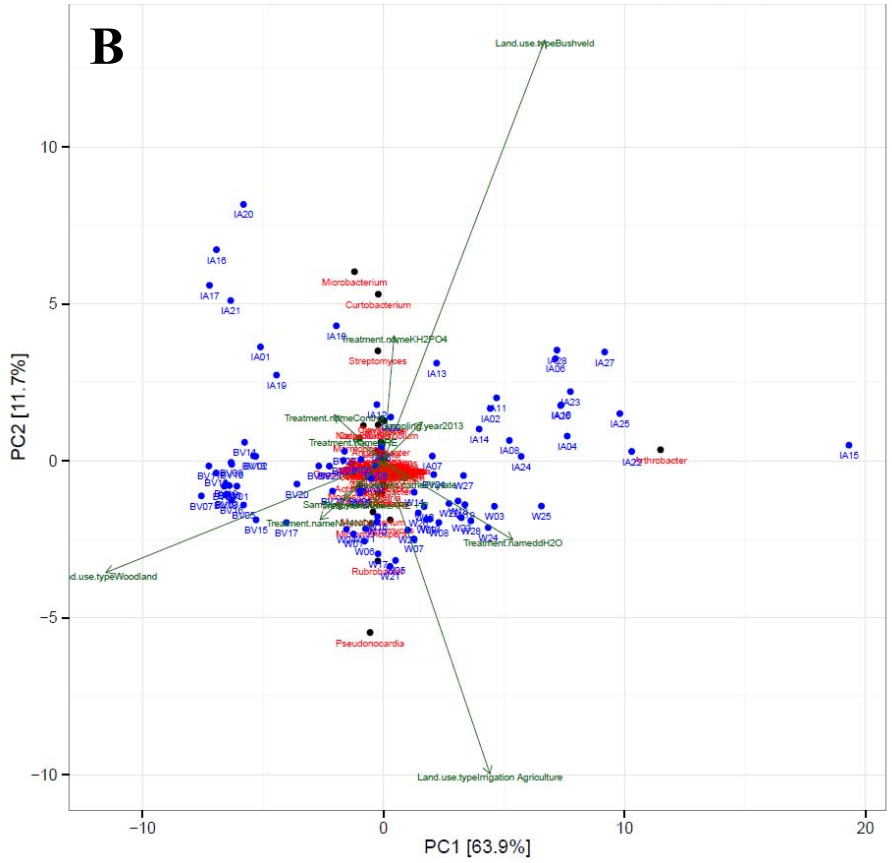


Figure 5: Unconstrained Principal Component Analysis based on non-scaled values of relative abundance of phyla (red) across soil samples (blue; W = woodland, BV = bushveld, IA = irrigation agriculture). The categorial factors of three stimulation parameters (year, Land use, treatment; darkgreen) were plotted posthoc. The PCA explains 97.8% of the variation.


A
PC2 [31.2%]
PC1 [50.1%]
Land.use.typeBushveld
Land.use.typeIrrigation Agriculture
Land.use.typeWoodl
Treatment.nameKH2PO4
Treatment.nameNH4NO3
Treatment.nameddH2O
Sampling.year2013
Gp3
Gp4
Gp6
Gp7
Gp16


B
PC2 [11.7%]
PC1 [63.9%]
Land.use.typeBushveld
Land.use.typeIrrigation Agriculture
Microbacterium
Curtobacterium
Streptomyces
Pseudonocardia
Arthrobacter
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Sampling.year2013

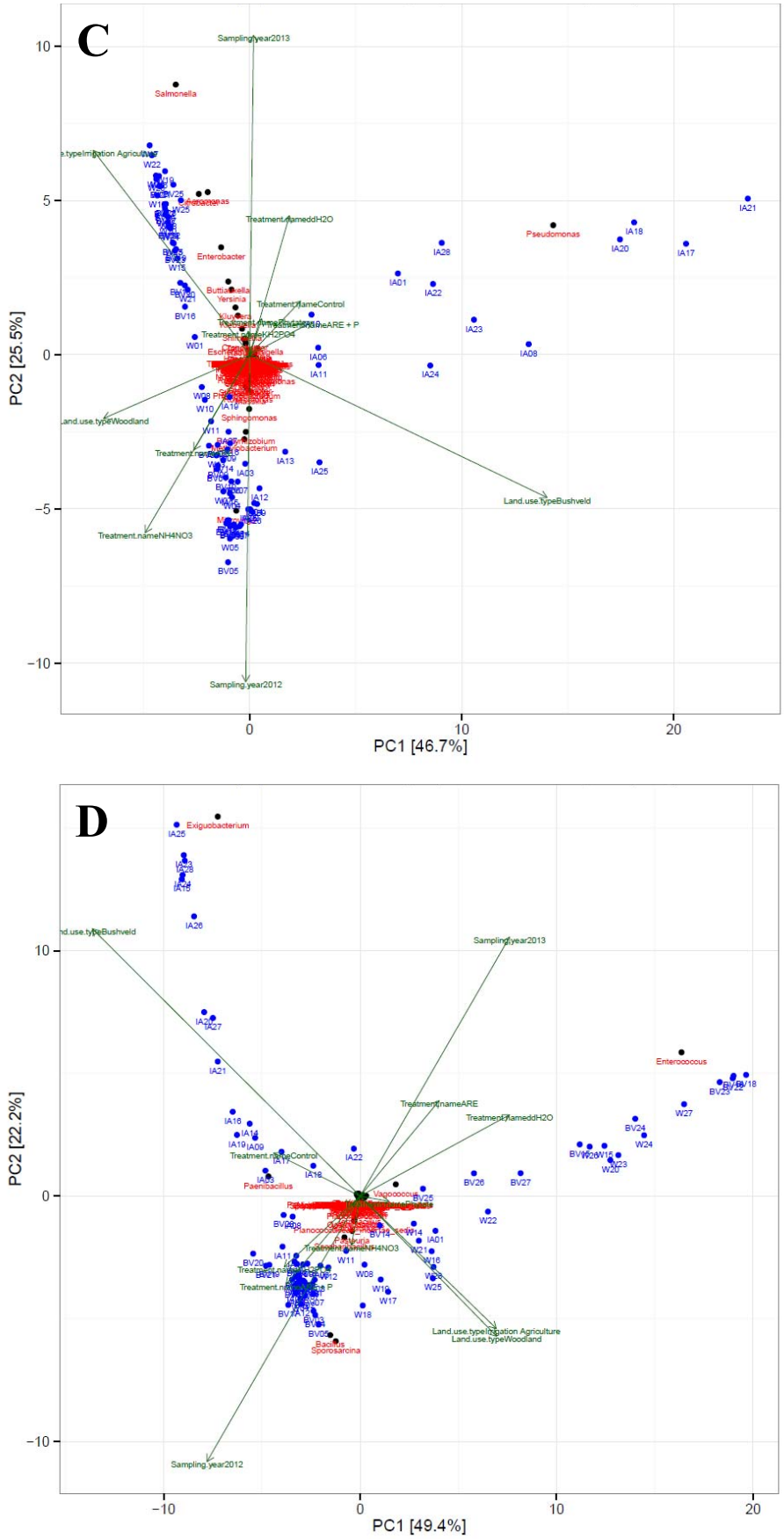

Figure 6: Unconstrained Principal Component Analysis based on non-scaled values of relative abundance of **A.** *Acidobacteria* genera, **B.** *Actinobacteria* families, **C.** order of *Proteobacteria* and **D.** *Firmicutes* families, (all red) across soil samples (blue; W = woodland, BV = bushveld, IA = irrigation agriculture). The categorial factors of three stimulation parameters (year, Land use, treatment; darkgreen) were plotted posthoc. The PCA explains **A.** 81.3%, **B.** 75.6%, **C.** 72.2% and **D.** 71.6% of the variation.

4.6. Discussion

4.6.1. Nutrient cycling in low fertility subtropical savannah soils

Namibian subtropical savannah soils are exposed to limitation of the major nutrients carbon, nitrogen and phosphorus (Gröngröft *et al*., 2013). However, nutrient cycling takes place in these soils. In the present study the activities of enzymes degrading complex carbon (β-glucosidase, β-xylosidase) and nitrogen (leucine aminopeptidase) compounds correlated with the amount of total carbon and total nitrogen, respectively. According to a C:N ratio of 10.7±0.88 determined in all ten Mashare soils, higher degradation activities of complex carbon than nitrogen compounds were examined in the majority of the soils. Beside the importance of carbon as part of microbial cells microorganisms respire carbon compounds in their metabolism. Therefore, high activities of enzymes degrading carbon compounds are necessary to gain carbon in the low fertility savannah soils. However, nitrogen is needed for the segregation of enzymes and for growth as it is part of amino acids. In the subtropical savannah soils low total nitrogen contents indicate that beside aminopeptidase activities nitrogen is provided by another pathway to the soil community. High activity abundances of nitrogen fixers like *Arthrobacter* (Sellstedt *et al.,* 2013) and *Paenibacillus* (von der Weid *et al.*, 2002) determined within the stimulation experiment suggest nitrogen fixation as a second considerable nitrogen providing pathway in subtropical savannah soils.

Nitrogen provided by the degradation of complex compounds or nitrogen fixation is further transformed by ammonification and nitrification. The analysis of degradation potential and nitrogen turnover rates revealed a correlation of exoenzyme activities and ammonification and nitrification rates. Illumina sequences revealed high abundances of soil degrading microorganisms like *Actinobacteria* (Rinkes *et al.,* 2013) and *Firmicutes*. Hence, amino compounds were provided which in turn were transformed by ammonification and nitrification and resulted in increased nitrogen transformation rates in the examined soils. Interestingly, ammonification and nitrification rates were not balanced in the majority of the examined soils. In the riparian woodland and bushveld savannah soils nitrification reached five to ten times higher values than ammonification. During the nitrogen turnover measurements the ammonium was probably adsorbed to soil particles (Blume *et al.*, 2010) and unavailable for the soil microbial community. However, low ammonium contents were determined in these pristine soils despite the addition of ammonium chloride during the field incubation. This indicated direct uptake of ammonium in the microbial or plant biomass or fast transformations of present ammonium by nitrification. The latter was confirmed by the examined high nitrification rates in the woodland and bushveld savannah soils. Due to nearly

undetectable *in situ* denitrification rates in the Mashare soils (per. comm. with Barbara Reinhold-Hurek) the liberated nitrate is either assimilated by the soil community, transformed to ammonium (which is in turn assimilated or used for nitrification) or leached during raining events, as nitrogen is not adsorbed by soil particles (Blume *et al.*, 2010). Hence, high nitrification rates in the rainy season might lead to a loss of nitrogen in the inherently low fertility subtropical savannah soils of Namibia.

4.6.2. Environmental parameters effecting degradation potential and nitrogen turnover rates in subtropical savannah soils

Several environmental parameters like soil temperature, moisture (Baldrian *et al.*, 2013; Rinkes *et al.*, 2013), SOM content, pH and cultivation (Waldrop *et al.*, 2000) alter the composition of the soil microorganisms community (Carreiro *et al.*, 2000; Treseder *et al.*, 2012). Microbial activities show fast response to external disturbances (Dick, 1994), i. e. fertilization (Lovell *et al.*, 1995), burning, thinning, ploughing or combination (Dick *et al.*, 1988; Concilio *et al.*, 2006; Gupta & Germida, 1988) metals or organic xenobiotics (Kandeler *et al.*, 1999b; Baldrian *et al.*, 2009).

In subtropical savannah soils both land use type and water availability effect the activity patterns of the soil microbial community. In soils with a low impact of human activities like the riparian woodland and bushveld savannah soils the activities of the microbial community reached highest values (Dick, 1984; Dick, 1988; Frank *et al.*, 2006). Riparian woodland and bushveld savannah soils provide increased soil organic matter (SOM) content and thereby nutrients, water (Wang *et al.*, 2003; Schlesinger & Andrews, 2000; Cleveland *et al.*, 2007), stable soil aggregates/microhabitats (Kandeler *et al.*, 1999a; Kanazawa & Filip, 1986) for the soil microbial community. Pristine soils have a dense vegetation and high organic input in form of litter, roots and plant residues (Bolton *et al.*, 1985; Martens *et al.*, 1992; Goyal *et al.*, 1993). Furthermore, the presence of roots restrains water and nutrients by adhesion forces and stimulates the soil microbial community by rhizodeposition (Hinsinger *et al.*, 2009; Jones *et al.*, 2009). High contents of SOM stimulate the inducible enzymes β-glucosidase, β-xylosidase and phosphatase excreted by microorganisms in subtropical savannah soils and explain the high exoenzyme activity values determined in soils dominated by a dense vegetation like the riparian woodland and bushveld savannah soils.

The activities of the soil microbial community decreased with increasing anthropogenic impact. Agriculturally used fields are disturbed by overexploitation and alteration of soil bulk density and soil structure (Johnson *et al.*, 1991) negatively effecting microbial TCN and the community composition and thereby activity values. Fertilization (Goyal *et al.*, 1993)

acidifies soils, alters the ionisation and solubility of enzymes, substrates, cofactors, stability of protein structures, organic xenobiotics and metals. Metals might act as inhibitors as metals mask catalytically active groups and denaturize or compete with cofactors (Kandeler *et al.*, 1999b; Baldrian *et al.*, 2009).

The water stress after the prolonged dry season of 2013 lysed the majority of the microbial cells, reduced the bacterial community or decreased the activities by the formation of duration form like spores. The drought caused low water availability and inhibited microbial activity by lowering intracellular water potential, by restricting substrate supply (Stark *et al.*, 1995) and inhibiting substrate enzyme interactivity resulting in lower nitrogen turnover rates. Interestingly, the riparian woodland soils still showed high TCN but clearly decreased activity values. The increased soil organic matter in the pristine soils prevented the microbial community by the provision of water and nutrients.

4.6.3. Environmental parameters effecting the composition of the active soil microbial community in subtropical savannah soils

Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes and *Firmicutes* represent typical bacterial phyla in soils (Janssen, 2006). But the composition of the active microbial community is varying with (a)biotic fluctuations of the environmental parameters. Both land use type and water availability effected the soils of the stimulation experiment investigated in this study. The examined Namibian soils were dominated by the *Actinobacteria*, *Proteobacteria* and *Firmicutes*. Moreover, only few prevailing species of these three typical soil phyla effected the activity patterns in the stimulated soils. Especially microorganisms which were adapted to nutrient limitation and water stress survived the harsh conditions of the subtropical savannah soils during the stimulation experiment. But the anthropogenic impact on soils like cultivation, fertilization and ploughing is effecting the composition of active soil microbial community as well. Cultivation and ploughing destroy microhabitats in the soils and compress the bulk soils. The addition of fertilizers reduced abundances and the diversity of soil microorganisms and lead to a shift to *Firmicutes* surviving increased fertilizer concentrations (i. e., Zn, Cu), whereas *Actinobacteria* and *Proteobacteria* showed increased activity parameters and abundances in riparian woodland and bushveld savannah soils.

Actinobacteria are optimal decomposers of complex soil organic matter (SOM; Rinkes *et al.*, 2013). Their mainly filamentous growth enables the infiltration of SOM and the degradation of complex compounds by exoenzymes (Větrovský *et al.*, 2014). Therefore, high activity abundances of this phylum are related with the SOM content and the input of organic

carbon by the present vegetation like the riparian woodland and bushveld savannah soils. Within the stimulation experiment *Arthrobacter* was the most abundant *Actinobacteria* genus. *Arthrobacter* is an ubiquitous soil organisms and well adapted to nutrient and water limitation in the examined Namibian soils (Boylen, 1973; Dworkin & Falkow, 2006; Hagedorn & Holt, 1975; Mongodin *et al.,* 2006). Beside the adaptation to water stress and heat, the *Arthrobacter* genus is capable of the degradation of complex soil compounds and even of pollutants. Hence, high abundances of *Arthrobacter* in woodland soils with a high SOM contents and in irrigated and fertilized fields are explained. The optimal adaptation to water stress and heat and the additional plasmids encoding for the degradation of complex soil compounds and even pollutants explains high activity abundances of the *Arthrobacter* in the Mashare soil samples.

After the rainy season the most abundant active *Firmicutes* were represented by ubiquitous genera *Bacillus* and *Sporosarcina*. In March 2013 the abundances of the active vegetative cells decreased and the microorganisms survived by the formation of inactive endospores due to drought and nutrient limitation. Despite the supplement with water and nutrients the endospores had not germinated. Either the rewetting period or amount of water was not sufficient or the supplemented nutrients had already been respired by other vegetative microorganisms like *Enterococcus*. Interestingly, *Enterococcus* predominantly inhabit the intestinal flora. But after the rainy season they dominated the activity patterns of the *Firmicutes* in the riparian woodland and in some bushveld savannah soils. The *Enterococcus* species of the stimulation experiment soils might originate from the local fauna or from cattle living in the riparian woodland or using the bushveld savannah soils as meadow, respectively. Although the study sites were protected by *Acacia* branches to prevent animal activities, wind probably transferred soils with the *Enterococcus* to the study sites. However, *Enterococcus* is adapted to strong variations in the environment as this genus is a typical intestinal inhabitant and naturally leaves this habitat from time to time. Hence, *Enterococcus* is able to survive soil conditions for a longer time and respond to rewetting and nutrient supplements fast. The soil genus *Paenibacillus* forming endospores and closely related to *Bacillus* prevailed in the rest of the dry bushveld savannah soils and some irrigated fields. Therefore, the land use type effected the composition of the active *Firmicutes* as well. Beside *Bacillus, Paenibacillus* and *Sporosarcina*, which are well adapted to different soil conditions and characterized by high degradation potential of different complex compounds, high abundances of the *Firmicutes* in the stimulation agriculture soils were mainly driven by *Exiguobacterium*. *Exiguobacterium* is well adapted to the conditions in the irrigated fields which are regularly fertilized with a

mixture of Cu, Zn, Fe and borax since this genus contains plasmids probably encoding for the degradation of soil organic matter or metal stress response (Vishnivetskaya *et al.,* 2009).

Within the *Proteobacteria* the most interesting patterns were influenced by the water availability on the active soil microbial community. After the rainy season the *Rhizobiales* as typical soil bacteria dominated the soils of the stimulation experiment. In contrast, after the prolonged dry season the activity abundance levels of the *Enterobacteriales* tremendously increased in the riparian woodland and bushveld savannah soils. The increased abundance levels of the *Enterobacteriales* might be caused by the presence of cattle and wild living animals in this area. Low activity levels of *Enterobacteriales* in the irrigated fields which were surrounded by a fence and protected against wild animals and grazing cattle confirm this hypothesis. However, *Enterobacteriales* are badly adapted to high concentrations of metals and fertilizers in soils and thereby the activity levels of these bacteria are also decreased in the irrigated and fertilized soils.

Interestingly, although *Acidobacteria* constitute up to 77% percent of the soil microbial community, the stimulated soils showed only low activity values of this phylum. Despite the low activity abundances, the *Acidobacteria* were further analyzed due to their possible importance in the nutrient cycling and due to the lack of knowledge of this ubiquitous phylum. The land use type and the water availability effected the composition of the *Acidobacteria* phylum and of subdivision 6 *Acidobacteria*. Subdivision 6 and 16 *Acidobacteria* are well adapted to the fertilization and the maize exudates in the irrigation agriculture soils and show increased abundances. Possibly genomes or additional plasmids encoding for the degradation of pollutants or soil exudates provide growth advantages of these subdivisions. High abundances of subdivision 6 *Acidobacteria* in the woodland and bushveld savannah soils where high exoenzyme activities and nitrogen turnover rates were detected indicate a participating in the nutrient cycling. The RDP database confirms that subdivision 6 *Acidobacteria* are the most abundant representatives of this phylum (George *et al.,* 2011). However, until now no isolate and no information about their role in the environment are available. Decreased abundances of subdivision 3 *Acidobacteria* in dry woodland and bushveld savannah soils indicate bad adaptation of these bacteria to dry conditions. In the irrigated soils this subdivision survived due to permanent water availability and possible adaptation to high fertilizer concentrations. In contrast, *Acidobacteria* subdivision 4 are well adapted to drought and nutrient limitation in subtropical savannah soils. The formation of thick cell walls like similar to the *Aridibacter* genus deriving from dry Namibian soils protects the vegetative cells against water stress (Huber *et al.,* 2014). The

presence of carotinoids as detected for *Aridibacter famidurans* counteracts radical oxygen species caused by increased UV radiation and prevents the cells before breaks of the DNA double strand (Huber *et al.,* 2014).

4.6.4. Soil relevant nutrients effecting the composition of the active soil microbial community in subtropical savannah soils

Arenosols contain very low or even undetectable amounts of the nutrients phosphorus and nitrogen. Both nutrients are essential for animals, plants and microorganisms as they are part of amino acids, enzymes, nucleic acids or the energy transporting compound ATP (adenosine triphosphate). The liberation of nitrogen and phosphorus by exoenzyme activities and further degradation/transforming steps (i. e. ammonification and nitrification) predominantly supports the metabolisms of fast growing microorganisms with a high demand of nitrogen and phosphorus like *Proteobacteria* (Gusewell & Freeman, 2005). Hence, the *Proteobacteria* originating from cattle and wild animals in the riparian woodland and bushveld savannah soils utilize the supplemented nutrients during the stimulation experiment after the dry season very fast. In contrast, other bacteria enduring in inactive cell forms are unable to answer to nutrient addition pulse in a short time.

However, beside the water stress adaptation mechanisms the prevailing bacterial species in the stimulation experiment soils have also evolved different methods for surviving nutrient limitation in the subtropical savannah soils. Different species of the *Paenibacillaceae*, *Rubrobacter* and *Arthrobacter* (Sellstedt *et al.,* 2013) fix nitrogen directly from air and survive nitrogen limitation in the stimulation soils. Interestingly, *Paenibacillus brasiliensis* a nitrogen fixer from the *Paenibacillaceae* is affiliated in the rhizosphere of maize explaining the high activity abundances of the *Paenibacillaceae* in the irrigation agriculture soils (von der Weid *et al.*, 2002). At both sampling time points maize was planted on the respective area.

4.7. Conclusion

Subsaharan savannah soils preserve habitats for various and rare animals, plants and microorganisms. Without sustainable land use management low soil fertility and an inadequate soil recovery capability of Subsaharan savannah soils lead to a continuous conversion of subtropical forests to farmland. Therefore, the unique biodiversity is endangered and the percentage of arid/semiarid soil surface is continuously increased. The present study confirmed that the composition and the activity of the microbial community and therefore the nutrient cycling in subtropical savannah soils are predominantly driven by the land use type and the availability of water. Anthropogenic impact on soils and water stress reduces the abundances and the variability of soil microorganisms. Furthermore, microorganisms adapted to the limiting conditions of the nutrients nitrogen and phosphorus have growth advantage in Namibian soils. The gained knowledge about the interdependencies of the microbial community and geochemical processes supports the development of sustainable land use management to obtain increased harvest crops.

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4.10. Supplementary Tables

Supplementary Table S1: Overview over the sample combination obtained during the stimulation experiment.

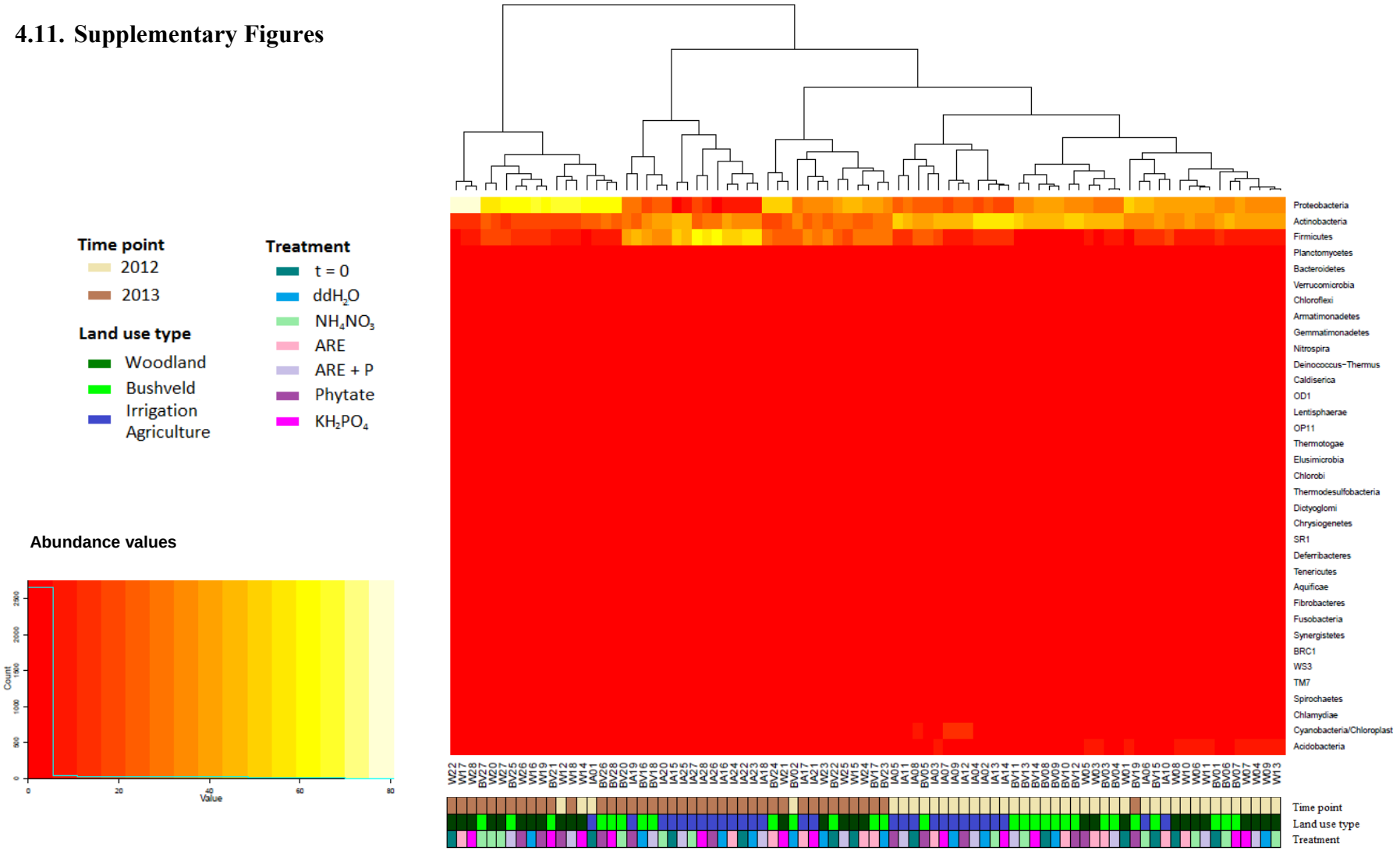
Woodland	Bushveld	Irrigation Agriculture	Time point	Parallel	Treatment
W01	BV01	IA01	2012	1	t = 0
W02	BV02	IA02	2012	1	ddH_2O
W03	BV03	IA03	2012	1	ARE
W04	BV04	IA04	2012	1	ARE + P
W05	BV05	IA05	2012	1	Phytate
W06	BV06	IA06	2012	1	NH_4NO_3
W07	BV07	IA07	2012	1	KH_2PO_4
W08	BV08	IA08	2012	2	t = 0
W09	BV09	IA09	2012	2	ddH_2O
W10	BV10	IA10	2012	2	ARE
W11	BV11	IA11	2012	2	ARE + P
W12	BV12	IA12	2012	2	Phytate
W13	BV13	IA13	2012	2	NH_4NO_3
W14	BV14	IA14	2012	2	KH_2PO_4
W15	BV15	IA15	2013	1	t = 0
W16	BV16	IA16	2013	1	ddH_2O
W17	BV17	IA17	2013	1	ARE
W18	BV18	IA18	2013	1	ARE + P
W19	BV19	IA19	2013	1	Phytate
W20	BV20	IA20	2013	1	NH_4NO_3
W21	BV21	IA21	2013	1	KH_2PO_4
W22	BV22	IA22	2013	2	t = 0
W23	BV23	IA23	2013	2	ddH_2O
W24	BV24	IA24	2013	2	ARE
W25	BV25	IA25	2013	2	ARE + P
W26	BV26	IA26	2013	2	Phytate
W27	BV27	IA27	2013	2	NH_4NO_3
W28	BV28	IA28	2013	2	KH_2PO_4

Abbreviations: BV, bushveld; IA, irrigation agriculture; and W, woodland.

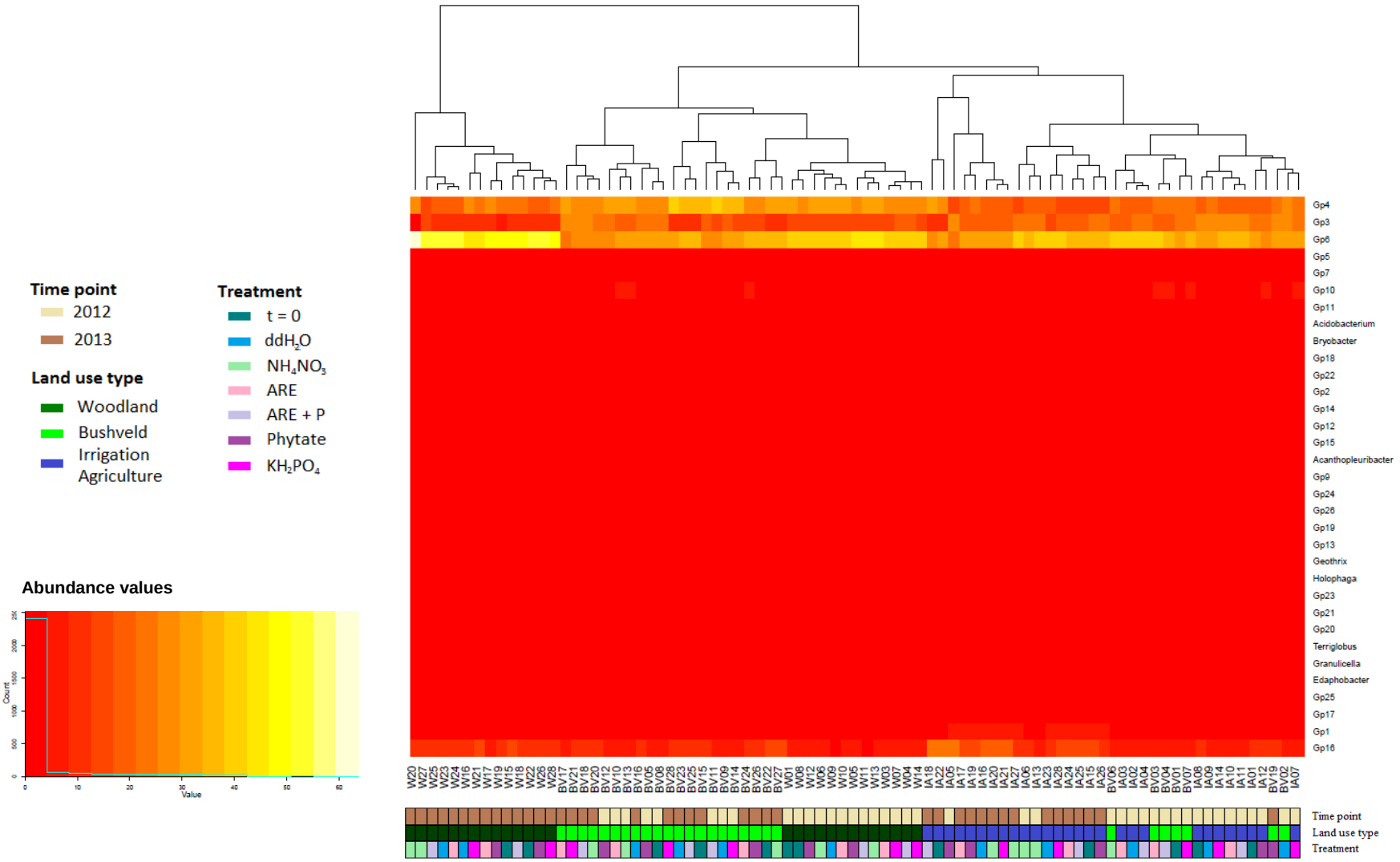
Supplementary Table S2: Composition of ARE (artificial root exudates) supplement solution as carbon source for stimulation experiment based on (Kraffczyk *et al.*, 1984; Kozdrój *et al.*, 2000) and adapted to the nutrient poor Subsaharan savannah soils.

Sugar	g/l	Carboxylic acid	g/l	Carboxylic acid	g/l
Glucose	0.36	Oxalic acid	0.005	Succinic acid	0.011
Arabinose	0.12	Fumaric acid	0.12	Benzoic acid	0.007
Fructose	0.12	Malic acid	0.010	Tartaric acid	0.003
Sucrose	0.15	Citric acid	0.025	Glutaric acid	0.001

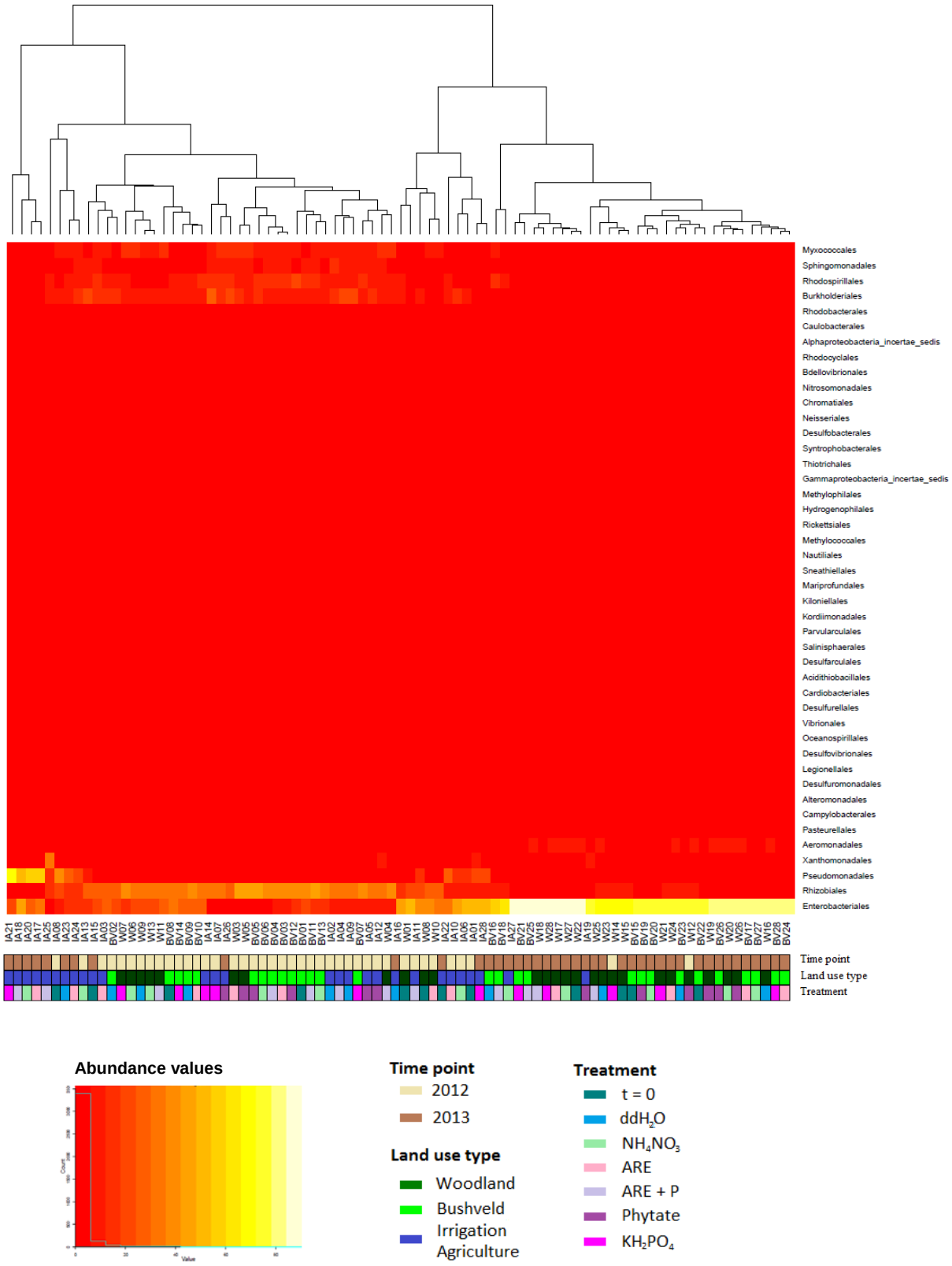
4.11. Supplementary Figures



Supplementary Figure S1: Heatmap of the relative abundance of phyla in Mashare, Namibia, in comparison to stimulation treatment, land use type, and time point of sampling. Time point: brown, 2012 after rainy season and dark brown, 2013 after prolonged dry season. Land use type: dark green, woodland (W); green, bushveld (BV) and irrigated fields (IA). Treatment: dark cyan, t=0; blue, ddH_2O; green, NH_4NO_3; rose, ARE; lavender, ARE + P; magenta, phytate; pink, KH_2PO_4. Abundance: from red, low values to yellow, high values.

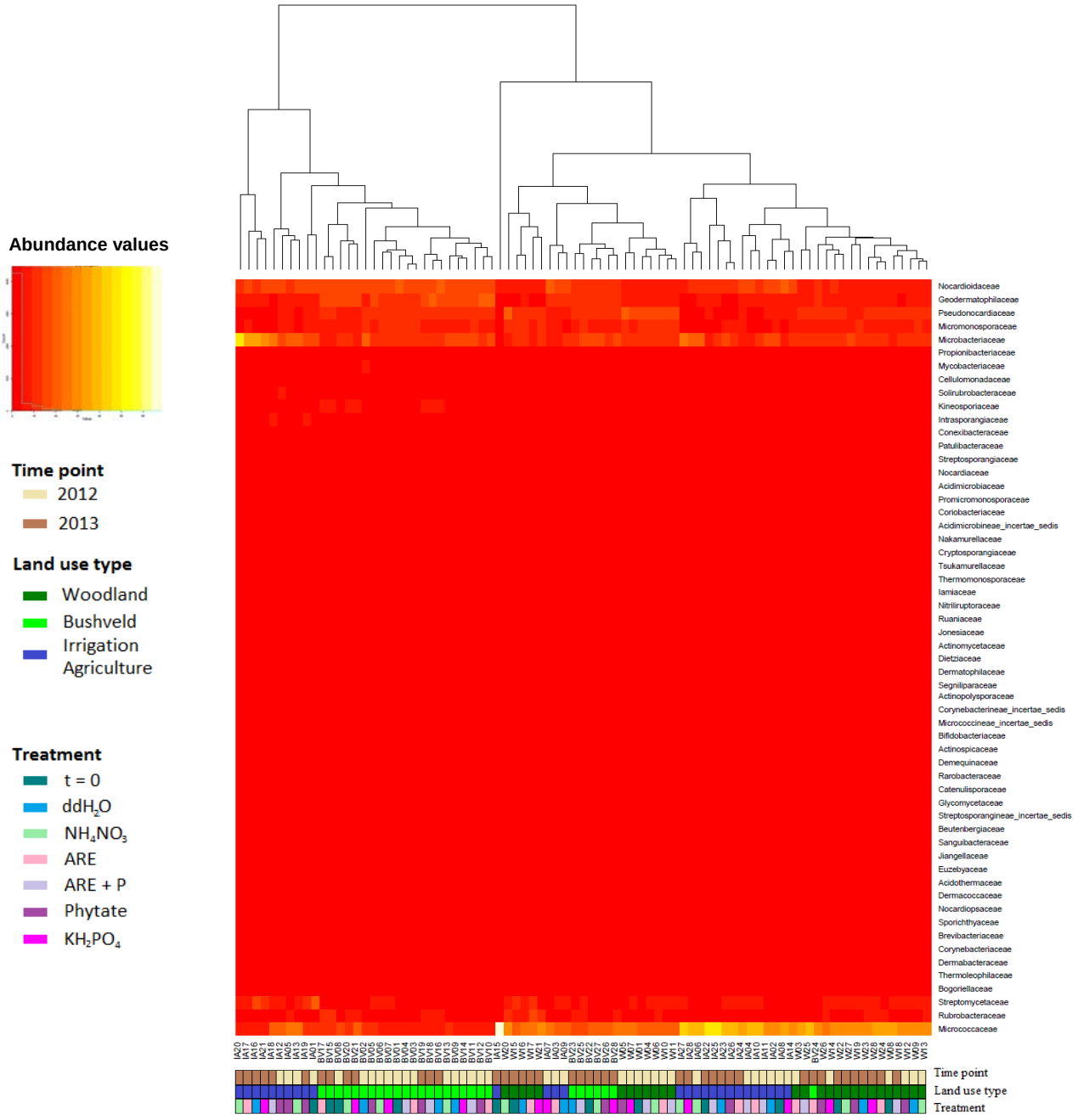


Supplementary Figure S2: Heatmap of the relative abundance *Acidobacteria* genera in Mashare, Namibia, in comparison to stimulation treatment, land use type, and time point of sampling. Time point: brown, 2012 after rainy season and dark brown, 2013 after prolonged dry season. Land use type: dark green, woodland (W); green, bushveld (BV) and irrigated fields (IA). Treatment: dark cyan, t=0; blue, ddH_2O; green, NH_4NO_3; rose, ARE; lavender, ARE + P; magenta, phytate; pink, KH_2PO_4. Abundance: from red, low values to yellow, high values.



Supplementary Figure S3: Heatmap of the relative abundance *Proteobacteria* order in Mashare, Namibia, in comparison to stimulation treatment, land use type, and time point of sampling. Time point: brown, 2012 after rainy season and dark brown, 2013 after prolonged dry season. Land use type: dark green, woodland (W); green, bushveld (BV) and irrigated fields (IA). Treatment: dark cyan, t=0; blue, ddH_2O; green, NH_4NO_3; rose, ARE; lavender, ARE + P; magenta, phytate; pink, KH_2PO_4. Abundance: from red, low values to yellow, high values.

Supplementary Figure S4: Heatmap of the relative abundance *Firmicutes* family in Mashare, Namibia, in comparison to stimulation treatment, land use type, and time point of sampling. Time point: brown, 2012 after rainy season and dark brown, 2013 after prolonged dry season. Land use type: dark green, woodland (W); green, bushveld (BV) and irrigated fields (IA). Treatment: dark cyan, t=0; blue, ddH_2O; green, NH_4NO_3; rose, ARE; lavender, ARE + P; magenta, phytate; pink, KH_2PO_4. Abundance: from red, low values to yellow high values.



Supplementary Figure S5: Heatmap of the relative abundance of *Actinobacteria* family in Mashare, Namibia, in comparison to stimulation treatment, land use type, and time point of sampling. Time point: brown, 2012 after rainy season and dark brown, 2013 after prolonged dry season. Land use type: dark green, woodland (W); green, bushveld (BV) and irrigated fields (IA). Treatment: dark cyan, t=0; blue, ddH_2O; green, NH_4NO_3; rose, ARE; lavender, ARE + P; magenta, phytate; pink, KH_2PO_4. Abundance: from red, low values to yellow high values.

Chapter 5

Aridibacter famidurans and *Aridibacter kavangonensis*, 2 novel species of *Acidobacteria* subdivision 4 isolated from semiarid savannah soil

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5.1. Contribution of the authors

Strain A22_HD_4H^T and strain Ac_23_E3^T were isolated by Bärbel Fösel/Susanne Mayer and Pia Wüst/Alicia Geppert, respectively. The physiological characterization of the strains A22_HD_4H and Ac_23_E3 like substrate tests, pH, temperature, salt tolerance, phase-contrast microscopy, sporulation, capsule and Gram staining and cell size determination were conducted by Katharina Huber with the assistance of Alicia Geppert. Katharina Huber and Alicia Geppert obtained the biomass production for the DNA-DNA hybridization, the determination of the fatty acids, quinone, G + C content which were examined by Gabriele Pötter and Birgit Grün. The cells for electron micrographs were grown and fixed by Katharina Huber, embedded by Ina Schleicher and the pictures were taken by Manfred Rohde. The figures and tables were conducted by Katharina Huber and the phylogenetic analysis and tree were performed by Bärbel Fösel. Katharina Huber and Bärbel Fösel wrote the article with the support of Jörg Overmann and Pia Wüst.

5.2. Abstract

Acidobacteria constitute an abundant fraction of the soil microbial community and are currently divided into 26 different subdivisions. Most cultivated *Acidobacteria* affiliate with subdivision 1, while only very few representatives of subdivisions 3, 4, 8, 10, and 23 have been isolated and described so far. Two novel isolates of *Acidobacteria* subdivision 4 were isolated from subtropical savannah soils and are characterized in the present work. Cells of strains A22_HD_4H^T and Ac_23_E3^T were immotile rods that divided by binary fission. Colonies were pink and white in color, respectively. The two novel *Acidobacteria* strains A22_HD_4H^T and Ac_23_E3^T were aerobic mesophiles with a broad range of tolerance towards pH (4.0-9.5 and 3.5-10.0, respectively) and temperature (15-44°C and 12-47°C, respectively). Both showed chemoorganoheterotrophic growth on some sugars, the amino sugar N-acetylgalactosamine, few amino acids, organic acids, and different complex protein substrates. Major fatty acids of A22_HD_4H^T and Ac_23_E3^T were $C_{15:0}$ iso, summed feature 1 ($C_{13:0}$ 3-OH/$C_{15:1}$ iso H), summed feature 3 ($C_{16:1}$ ω7c/$C_{16:1}$ ω6c), and $C_{17:0}$ anteiso. The major quinone was MK-8. In addition, MK-7 occurred in small amounts. The DNA G+C content of A22_HD_4H^T and Ac_23_E3^T was 53.2 and 52.6 mol%, respectively. The closest described relative was *Blastocatella fastidiosa* A2-16^T with a 16S rRNA gene sequence identity of 93.2 and 93.3%, respectively. Strains A22_HD_4H^T and Ac_23_E3^T displayed a 16S rRNA gene sequence similarity of 97.4% to each other. According to the low DNA-DNA hybridization value, the two isolates represent two different species. Based on morphological, physiological, and molecular characteristics, the novel genus *Aridibacter* gen. nov., including two novel species, *A. famidurans* sp. nov. strain A22_HD_4H^T (= DSM 26555^T = LMG 27985^T) and *A. kavangonensis* sp. nov. strain Ac_23_E3^T (= DSM 26558^T = LMG 27597^T) are proposed.

5.3. Introduction, Material and Methods, Results and Discussion

Over the last decades the majority of newly described bacterial species were representatives of the phyla *Actinobacteria, Firmicutes,* and *Proteobacteria* (Hugenholtz *et al.,* 1998; Janssen *et al.,* 2002). In contrast, only few *Acidobacteria* are validly described, although this phylum accounts for up to 70% of the soil microbial community (Janssen, 2006; Jones *et al.,* 2009; Lauber *et al.*, 2009) and is also abundant in other types of habitats such as hot springs (Hobel *et al.,* 2005), polar deserts of Antarctica (Pointing *et al.,* 2009), wastewater (LaPara *et al.,* 2000), and cave paintings (Schabereiter-Gurtner *et al.,* 2004). The *Acidobacteria* are divided into 26 different subdivisions (Barns *et al.,* 1999) and only subphyla 1, 3, 4, 8, 10, and 23 contain at least one described species. Eight genera [*Acidobacterium* (Kishimoto *et al.,* 1991), *Terriglobus* (Baik *et al.*, 2013; Eichorst *et al.,* 2007; Männistö *et al.,* 2011; Whang *et al.*, 2014), *Edaphobacter* (Koch *et al.,* 2008), *Acidicapsa* (Kulichevskaya *et al.,* 2012), *Granulicella* (Männistö *et al.,* 2012; Pankratov & Dedysh, 2010), *Telmatobacter* (Pankratov *et al.,* 2012), 'Acidipila' (Okamura *et al.,* 2011), and *Bryocella* (Dedysh *et al.,* 2012)] belong to subdivision 1, one genus [*Bryobacter* (Kulichevskaya *et al.,* 2010)] belongs to subdivision 3, three genera [*Holophaga* (Liesack *et al.,* 1994), *Geothrix* (Coates *et al.,* 1999), and *Acanthopleuribacter* (Fukunaga *et al.,* 2008)] belong to subdivision 8, one genus ['Thermotomaculum' (Izumi *et al.,* 2012)] to subdivision 10, and one genus [*Thermoanaerobaculum*, (Losey, *et al*., 2013)] to subdivision 23. Subdivision 4 *Acidobacteria* are abundant in different soils (Barns *et al.*, 1999; Foesel *et al.*, 2014; Jones *et al.,* 2009) and were shown to be cultivable (Bryant *et al.*, 2007; George *et al*., 2011; Joseph *et al.*, 2003; Stott *et al.*, 2008). Recently the first two members of this subdivision, *Blastocatella fastidiosa* (Foesel *et al.*, 2013) and *Pyrinomonas methylaliphatogenes* (Crowe *et al.*, 2014), were validly described. The existence of the co-cultured strain *Candidatus* 'Chloracidobacterium thermophilum' (Bryant *et al.*, 2007) has been reported earlier. While *B. fastidiosa* and *P. methylaliphatogenes* are slightly acidophilic or neutrophilic chemoorganoheterotrophic soil bacteria (Crowe *et al.* 2014; Foesel *et al.*, 2013), *Candidatus* 'C. thermophilum' is a basophilic photoheterotroph thriving in microbial mats of hot springs (Bryant *et al.*, 2007). *B. fastidiosa* is a mesophile, the two other strains show thermophilic traits. In the present study, two novel isolates are described that extend the group of cultivated subdivision 4 *Acidobacteria*.

Strain A22_HD_4H^T was isolated from a clayey sand soil with slightly basic pH (7.4 and 8.2 measured in 2 mM $CaCl_2$ and in distilled water, respectively). The soil sample was collected in spring 2009 from a pasture at the farm Erichsfelde, central Namibia

(21°38´15.8´´S 16°52´03.9´´E, 1497 m height above sea level). Strain Ac_23_E3^T originated from a sandy fallow with a similar pH (7.3 and 8.2 measured in 2 mM $CaCl_2$ and in distilled water, respectively) sampled in spring 2011 at Mashare, Kavango region, in northern Namibia (17°53'37.93´´S 20°14'50.71´´E, 1069 m height above sea level). For the isolation of novel *Acidobacteria*, soil suspensions (10 mM HEPPS, pH 8.0) were inoculated in 200 µl of liquid SSE/HD 1:10 (Foesel *et al.*, 2013). Instead of MES, 10 mM HEPPS was used to buffer the SSE/HD medium at a pH of 8.0. After 6 weeks of incubation at 20°C, cultures were screened for acidobacterial growth by group specific PCR using the primer pair Acido31f (Barns *et al.*, 1999)/1492r (Lane, 1991). Cultures that yielded PCR products were plated on SSE/HD 1:10 plates solidified with purified agar (15 g l^{-1}; Oxoid, Basingstoke, UK). Strain Ac_23_E3^T was isolated by subsequent streaking on plates. In contrast, strain A22_HD_4H^T was only gained in pure culture by plating after a prolonged starvation period of an enrichment culture in pure soil solution equivalent (SSE; Angle *et al.,* 1991), pH 8.0. Unless otherwise noted SSE/HD 1:10 was also used in the following physiological tests and for biomass production.

Liquid cultures of strain A22_HD_4H^T displayed a yellow color in initial stages. With increasing density, cultures developed a bright pink color and formed aggregates even during shaking. On agar plates, colonies of A22_HD_4H^T had a diameter of 0.1-0.2 mm and were pink in color, circular, translucent, convex, and opaque with entire margins. Strain Ac_23_E3^T formed white colonies with a light pinkish hue. The colonies had a diameter of 0.2-0.3 mm and were translucent, convex, and opaque with entire margins. Liquid cultures of strain Ac_23_E3^T were white and did not form aggregates. Cell morphology of both strains was examined by light microscopy (Zeiss Axio Lab.A1, Carl Zeiss, Oberkochen, Germany). Pictures were taken with a Zeiss Axio Imager.M2 microscope (Carl Zeiss) equipped with an AxioCam MRm camera. Cells of both strains were rod shaped and divided by binary fission. Single cells of strains A22_HD_4H^T and Ac_23_E3^T were 2.5-3.0 µm long and about 0.9 µm and 0.6-0.7 µm in diameter, respectively. In contrast to cells of strain A22_HD_4H^T which formed single cells, strain Ac_23_E3^T additionally formed short chains of 2 to 4 cells (Fig. 1 A-D). However, formation of longer chains as found in the closest relative *B. fastidiosa* A2-16^T (Foesel *et al.,* 2013) was not observed. Consistent with all other characterized *Acidobacteria*, both strains stained Gram-negative (Gerhardt *et al.*, 1994). Capsule and spore formation was examined by India ink and malachite green staining, respectively (Bast, 2011), and was not observed for strains A22_HD_4H^T and Ac_23_E3^T which is similar to the properties of *B. fastidiosa* A2-16^T (Foesel *et al.*, 2013).

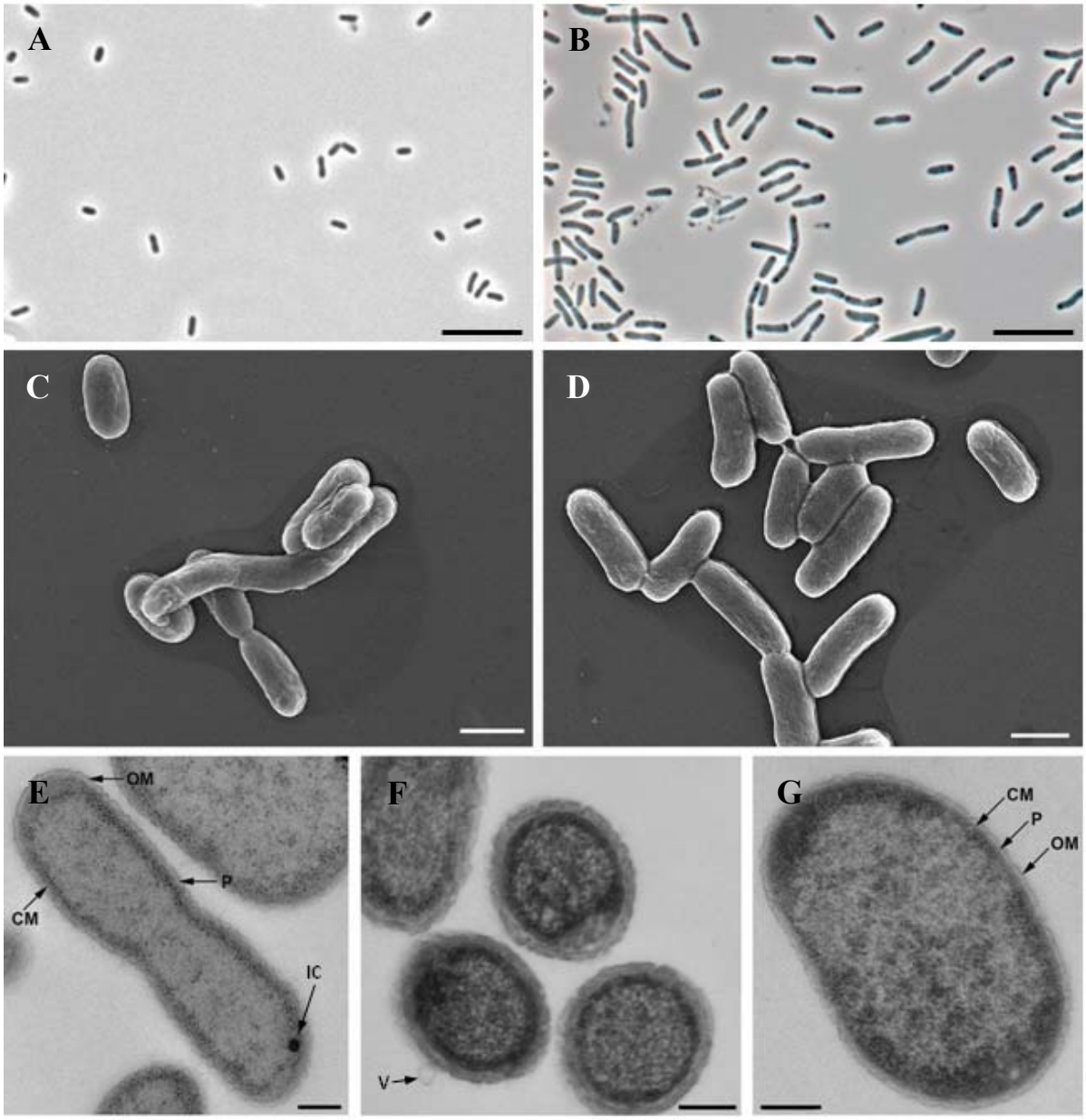

Figure 1. Phase-contrast photomicrographs of strains A22_HD_4H^T (**A**) and Ac_23_E3^T (**B**). Scale bar, 10 µm; scanning electron microscope pictures of strains A22_HD_4H^T (**C**) and Ac_23_E3^T (**D**). Scale bar, 1 µm; transmission electron micrographs of strain A22_HD_4H^T (**E-F**) and strain Ac_23_E3^T (**G**). Scale bar, 200 nm (**E-G**). OM = outer membrane, P = periplasma, CM = cytoplasmic membrane, IC = intracytoplasmic inclusion body, V = outer membrane vesicle.

For transmission electron microscopy cells were prepared as described earlier (Foesel *et al.,* 2013), however, using 1% (v/v) glutaraldehyde and 2% (v/v) formaldehyde in the initial fixation step. Both strains revealed the structure of a Gram-negative cell wall (Fig. 1 E-G). Older cultures (incubated for 4 weeks) showed thickened cell walls (about 30 nm; Fig. 1 F).

Several cells of the strains A22_HD_4H^T and Ac_23_E3^T contained intracytoplasmic inclusion bodies (Fig. 1 E). Outer membrane vesicles additionally occurred (Fig. 1 F).

Almost full-length 16S rRNA gene fragments of strains A22_HD_4H^T and Ac_23_E3^T were amplified and sequenced as described before (Foesel *et al.,* 2013). Sequences were added to the small subunit ribosomal RNA non-redundant reference database SILVA version

108 [www.arb-silva.de; (Pruesse *et al.,* 2007)] using the program package ARB (Ludwig *et al.,* 2004).

Phylogenetic trees were calculated using neighbor joining, maximum parsimony, and maximum likelihood algorithms (termini filter; 41,485 valid positions between position 60 and 1438 of the *Escherichia coli* 16S rRNA reference gene; 1000 bootstrap resamplings). All three methods placed the two novel strains A22_HD_4H^T and Ac_23_E3^T in the vicinity of *B. fastidiosa* A2-16^T (Foesel *et al.,* 2013) within subdivision 4 of the phylum *Acidobacteria* (93.2 and 93.3% nucleotide similarity, respectively; Fig. 2). To the two further subdivision 4 members *P. methylaliphatogenes* K22^T (Crowe *et al*., 2014) and *Candidatus* 'C. thermophilum' (Bryant *et al.*, 2007) the two novel stains were only distantly related (~ 83% nucleotide similarity). The closest phylogenetic relatives (96–98% nucleotide similarity) were some clone sequences derived from environments such as soils (AB637077, GU444118, both unpublished), soil crusts (JX255145; unpublished), subsurface sediments (HM186080; Lin *et al.,* 2012), skin (JF139787, JF175323; Kong *et al.,* 2012), and clean rooms (DQ532357; Moissl *et al.,* 2007) (Fig. 2). Strains A22_HD_4H^T and Ac_23_E3^T had a 16S rRNA gene sequence identity of 97.4%. This value is below the threshold of 98.7-99.0% 16S rRNA gene sequence identity for which DNA-DNA hybridization is recommended to be mandatory (Stackebrandt & Ebers, 2006). However, DNA-DNA hybridization was performed to confirm the existence of two different species following a more conservative threshold of 97% 16S rRNA gene identity for species definition and recommendation for additional DNA-DNA hybridization experiments (Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001).

The established protocol for DNA-DNA hybridization (De Ley *et al.,* 1970) was modified (Huss *et al.,* 1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with in situ temperature probe (Varian). Cells of strains A22_HD_4H^T and Ac_23_E3^T were disrupted in a Constant Systems TS 0.75 KW (IUL Instruments, Koenigswinter, Germany) and the DNA was purified in the crude lysate by chromatography on hydroxyapatite (Cashion *et al.,* 1977). Duplicate measurements yielded hybridization values of 29.8% and 23.7% for strains A22_HD_4H^T and Ac_23_E3^T that fall far below the limit for species definition of 70% (Wayne *et al*., 1987) and confirm the assignment of the two strains to two different species.

For G+C content determination cells were disrupted in a French press. The DNA was purified using hydroxyapatite (Cashion *et al.,* 1977). After sample treatment with P1 nuclease and analysis by high performance liquid chromatography (HPLC, Shimadzu Corporation,

Kyoto, Japan) the G+C content was calculated (Mesbah *et al.,* 1989; Tamaoka & Komagata, 1984) to be 53.2 and 52.6 mol% for strains A22_HD_4H^T and Ac_23_E3^T, respectively. These numbers fall within the range of G+C content of 51.6-62.7% known for any other established species of *Acidobacteria* subdivision 1, 3, 4, 8, 10, and 23, but clearly exceed the G+C content of 46.5 mol% of *B. fastidiosa* A2-16^T (Tab. 1).

Isoprenoid quinones were extracted from dried biomass with chloroform/methanol (2:1, v/v) (Collins & Jones, 1981) and analyzed via HPLC (Tindall, 1990). The detected quinones were MK-8 and in very small amounts MK-7 which is in congruence with the quinone composition of *B. fastidiosa* A2-16^T (Tab. 1; Foesel *et al.*, 2013) and the other described subdivision 4 *Acidobacteria* (Bryant *et al.*, 2007; Crowe *et al.*, 2014).

For fatty acid analysis, cells were grown in liquid SSE/HD 1:10 for 8 days at 28°C. Data taken from literature were obtained under growth conditions comparable to those used for strains A22_HD_4H^T and Ac_23_E3^T. All used media contained glucose or glucuronic acid and yeast or casamino acids (0.1-0.5 g l^{-1}) as growth factors. Protein substrates such as peptone (0.5 g l^{-1}) were also present. After harvesting about 40 mg wet weight, cells were extracted according to the standard protocol (Sasser, 1990) of the Microbial Identification System (MIDI Inc.; version 6.1) for fatty acid analysis. The composition of the fatty acids was identified by the comparison to TSBA40 peak naming table database. Strains A22_HD_4H^T and Ac_23_E3^T possessed straight chain, methyl and/or hydroxyl-branched unsaturated and monosaturated fatty acids. The major fatty acids of A22_HD_4H^T and Ac_23_E3^T were $C_{15:0}$ iso (35.1 and 38.0%), summed feature 3 ($C_{16:1}$ ω7c/$C_{16:1}$ ω6c, 20.1 and 15.3%), summed feature 1 ($C_{13:0}$ 3-OH/$C_{15:1}$ iso H, 17.2 and 11.7%), and $C_{17:0}$ anteiso (6.2 and 10.9%). Both strains additionally contained $C_{16:0}$ (3.6 and 5.0%), $C_{13:0}$ iso (4.1 and 3.5%), and $C_{17:1}$ anteiso A (3.0 and 4.1%). In summary fatty acid profiles of the two strains vary rather on percentage of single compounds than in their composition (Supplementary Tab. 1). High amounts of $C_{15:0}$ iso and summed feature 3 ($C_{16:1}$ ω7c/$C_{16:1}$ ω6c) are a feature strains A22_HD_4H^T, Ac_23_E3^T and further subdivision 1 members [*Granulicella* spp. (Pankratov & Dedysh *et al.*, 2010), *Bryocella elongata* SN10^T (Dedysh *et al.*, 2012), *Terriglobus roseus* KBS 63^T (Eichorst *et al.*, 2007), *Edaphobacter aggregans* Wbg-1^T (Koch *et al.*, 2008)] have in common. Yet, the high content of summed feature 1 ($C_{13:0}$ 3-OH/$C_{15:1}$ iso H) and the presence of $C_{17:0}$ anteiso distinguishes the novel strains from these *Acidobacteria* representatives. High amounts of summed feature 1 ($C_{13:0}$ 3-OH/$C_{15:1}$ iso H), summed feature 3 ($C_{16:1}$ ω7c/$C_{16:1}$ ω6c), and the presence of $C_{17:1}$ anteiso A in strains A22_HD_4H^T and Ac_23_E3^T are in congruence with the fatty acid profile of *B. fastidiosa* A2-16^T (Tab. 1).

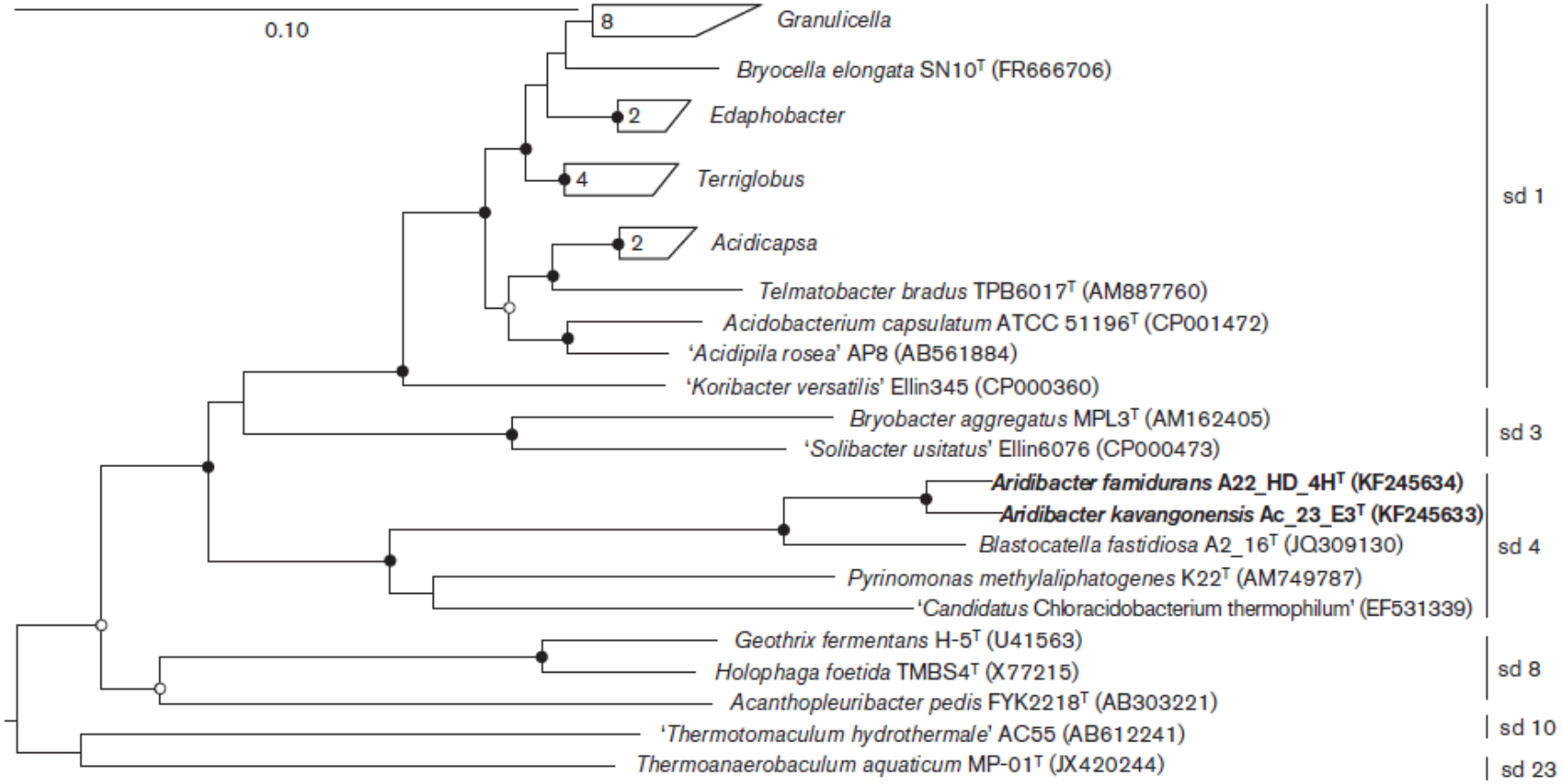

Figure 2. Rooted neighbor-joining phylogenetic tree (Felsenstein correction) based on almost full-length 16S rRNA gene sequences showing the relationship of the strains A22_HD_4H^T and Ac_23_E3^T and related taxa. Open and closed circles indicate bootstrap values (expressed as a percentages of 1000 replicates) of ≥ 70% and ≥ 90%, respectively. The following sequences were used as outgroup: *Planctomyces brasiliensis* DSM5305^T (AJ231190) and *Planctomyces maris* DSM8797^T (AJ231184). Bar indicates 10% nucleotide divergence.

Table 1: Characteristics of A22_HD_4H^T and Ac_23_E3^T compared with the related type strain *Blastocatella fastidiosa* A2-16^T (Foesel *et al.*, 2013); cultivation conditions of A22_HD_4H^T, Ac_23_E3^T and *B. fastidiosa* A2-16^T were comparable.
Strains: 1, A22_HD_4H^T; 2, Ac_23_E3^T; 3, *B. fastidiosa* A2-16^T.
All strains utilized protocatechuate, casamino acids, casein hydrolysate, peptone, and yeast extract.
All strains derived from semiarid savanna soils, formed no spores and capsules, and contained MK-8 as major quinone.
+, positive; -, negative; (+), weak growth detected; ND, no data available.

Characteristics	1	2	3
Cell shape	Rod	Rod	Sphere to rod
Cell size (µm)	2.5-3.0 x 0.9	2.5-3.0 x 0.6-0.7	0.8-12.0 x 0.8-0.9
Cell division	Binary fission	Binary fission	Binary fission/ budding
Motility	-	-	+
Pigmentation	Yellow - pink	White (bright pinkish hue)	Orange - pink
NaCl tolerance (% w/v)	≤1.0	≤1.0	<1.0
Temperature range (°C)	15-44	12-47	14-40
Temperature optimum (°C)	24-36	36-44	29-35
pH range	4.0-9.5	3.5-10.0	4.0-10.0
pH optimum	5.5-9.0	5.5-8.0	5.0-7.5
DNA G+C-content (mol %)	53.2	52.6	46.5
Major fatty acids (%)			
$C_{13:0}$ iso	**4.1**	**3.5**	1.0
$C_{15:0}$ iso	**35.1**	**38.0**	5.0
$C_{15:1}$ iso H/$C_{13:0}$ 3-OH	**17.2**	**11.7**	**22.7**
$C_{16:0}$	**3.6**	**5.0**	0.6
$C_{16:0}$ iso	0.9	1.9	**15.7**
$C_{16:1}$ iso H	-	-	**4.6**
$C_{16:1}$ ω7c/$C_{16:1}$ ω6c	**20.1**	**15.3**	**25.3**
$C_{17:0}$ anteiso	**6.2**	**10.9**	3.0
$C_{17:1}$ iso ω9c	0.7	2.3	**9.8**
$C_{17:1}$ anteiso A	3.0	**4.1**	2.6
Carbon sources utilized			
Cellobiose	(+)	-	-
Lactose	-	(+)	-
Maltose	-	+	-
Rhamnose	+	+	-
Xylose	+	-	-

Asparagine	(+)	(+)	-
Glutamine	-	(+)	-
N-acetylgalactosamine	+	+	-
Heptanoic acid	(+)	-	-
Trimethoxybenzoate	-	(+)	-
Fumarate	-	+	-
Isovalerate	-	+	-
Laminarin	-	+	-
Chitin	-	(+)	(+)
Cellulose	-	+	(+)
Starch	-	(+)	(+)

Compared to *B. fastidiosa* A2-16^T strains A22_HD_4H^T and Ac_23_E3^T contain higher amounts of $C_{13:0}$ iso, $C_{15:0}$ iso, $C_{16:0,}$ and $C_{17:0}$ anteiso. In contrast, *B. fastidiosa* A2-16^T contains higher amounts of $C_{16:0}$ iso and $C_{17:1}$ iso ω9c (Tab. 1). A further distinctive feature is the occurrence of $C_{16:1}$ iso H in *B. fastidiosa* A2-16^T. The high amount of $C_{15:0}$ iso is a trait strains A22_HD_4H^T and Ac_23_E3^T have in common with the further subdivision 4 strain *P. methylaliphatogenes* K22^T, while the latter excels due to the high amounts of further saturated *iso*-branched fatty acids ($C_{17:0}$ iso, $C_{19:0}$ iso, $C_{21:0}$ iso; Crowe *et al.*, 2014).

The polar lipid composition of strains A22_HD_4H^T and Ac_23_E3^T was analyzed by the two-dimensional thin layer chromatography method (modified after Bligh & Dyer, 1959; Tindall *et al.;* 2007). Both strains contained phosphatidylcholine, phosphatidylglycerol, diphosphatidylcholine, and phosphatidylethanolamine. (Supplementary Fig. S1). Presence of phosphatidylethanolamine and phosphatidylglycerol has also been described for the *Acidobacteria* subdivision 1 species *Acidobacterium capsulatum* 161^T, *Telmatobacter bradus* TPB6017^T (Pankratov *et al.*, 2012), and for *Thermoanaerobaculum aquaticum* MP-01^T (Losey *et al.*, 2013). Dedysh *et al.* (2012) confirmed the presence of phosphatidylcholine in *A. capsulatum* 161^T and Kulichevskaya *et al.* (2012) determined phosphocholine in *Acidicapsa borealis* KA1^T and *A. capsulatum* 161^T. The existence of diphosphatidylcholine as determined for strains A22_HD_4H^T and Ac_23_E3^T was also reported for *T. aquaticum* MP-01^T (Losey *et al.*, 2013).

Growth ranges and optima of temperature and pH were determined in triplicates under oxic conditions in liquid SSE/HD 1:10 media. Temperature values between 10 and 56°C and pH values between 1.0 and 11.0 were tested. Depending on the respective pH value MES, HEPES, HEPPS, or CHES (Sigma-Aldrich, Steinheim, Germany or Applichem, Darmstadt, Germany, 10 mM each) were used as buffers. Growth was determined by measuring the

optical density at 660 nm. Growth of strain A22_HD_4H^T occurred between 15 and 44°C and between pH 4.0 and 9.0. Optimal growth (defined as ≥ 75% of highest growth rate achieved) was determined at 24-36°C (highest rate at 27°C) and pH 5.5-9.0 (highest rate at pH 7.0). Strain Ac_23_E3^T grew between 12 and 47°C and between pH 3.5 and 10.0. Optimal growth occurred at 36-44°C (highest rate at 39°C) and pH 5.5-8.0 (highest rate at pH 6.5). Salt tolerance was determined in HD 1:10 medium [0.25 g l^{-1} yeast extract, 0.5 g l^{-1} of peptone, 0.1 g l^{-1} glucose, 0.1 ml l^{-1} ten vitamin solution (Balch *et al.,* 1979), and 1 ml l^{-1} trace element solution SL 10 (Tschech & Pfennig, 1984)] with varying NaCl concentrations between 0 to 10% (w/v). Strains A22_HD_4H^T and Ac_23_E3^T tolerated concentrations of 0-1% (w/v) NaCl. They grew best at a NaCl concentration of 0.25% (w/v) while growth of *B. fastidiosa* A2-16^T was already inhibited at this NaCl concentration (Foesel *et al.*, 2013). The ability to tolerate a broad range of temperatures and pH values is comparable to that of *B. fastidiosa* A2-16^T (Tab. 1) and *P. methylaliphatogenes* K22^T (Crowe *et al.*, 2014) and thus might be an important feature of subdivision 4 *Acidobacteria*. Within the three mesophilic Namibian strains A22_HD_4H^T, Ac_23_E3^T, and *B. fastidiosa* A2-16^T, Ac_23_E3^T excels due to its elevated temperature optimum, whereas *P. methylaliphatogenes* K22^T stands out due to its thermophilic traits.

The range of growth substrates utilized by strains A22_HD_4H^T and Ac_23_E3^T was tested in two parallels of liquid oxic SSE/HD 1:10 medium. For this purpose peptone and glucose were omitted completely, yeast extract was added only in traces. The growth substrates tested comprised sugars, organic acids, keto acids, alcohols, amino acids (0.5 to 10 mM each; Supplementary Tab. 2), casamino acids, casein hydrolysate, laminarin, peptone, yeast extract (0.05% w/v each), and Tween 80 (0.001% w/v). Growth on cellulose (microcrystalline, 20 µm particle size, Sigma-Aldrich), chitin (from crab shells, Roth, Karlsruhe, Germany), and starch (soluble, Merck) was tested on solidified media with a final concentration of 0.5 g l^{-1} of the respective substrate. Additionally, a negative control was prepared without the addition of any polymer. After six weeks of incubation at 20°C degradation of starch, cellulose, and chitin was assessed by the appearance of colonies and by probing the presence of clear zones around colonies, in the case of starch and cellulose after staining with Lugol´s solution (Cowan, 1993) and Congo red (Wood & Bhat, 1988), respectively.

Cytochrome *c*-oxidase and catalase activities were determined by established protocols (Cowan *et al.,* 1993; Gerhardt, 1994). Cytochrome *c*-oxidase was additionally tested by Bactident® Oxidase (Merck, Darmstadt, Germany). Indol formation, aesculin degradation,

urease activity, and further exoenzyme activities were determined by the API ZYM and API 20 NE test systems (Biomérieux, Marcy l'Etoile, France). Similar to *B. fastidiosa* A2-16^T, the preferred growth substrates of strains A22_HD_4H^T and Ac_23_E3^T were protein containing, complex substrates such as casamino acids, peptone, or yeast extract as well as protocatechuate (Tab. 1). Yet, the two novel strains showed a slightly broader substrate range as they also grew on some sugars like the amino sugar N-acetylgalactosamine, a few amino acids, and organic acids. This is a feature shared with the further *Acidobacteria* subdivision 4 member *P. methylaliphatogenes* K22^T that showed an even broader range of substrate utilization (Crowe *et al.*, 2014). The two novel strains differed with respect to the type of sugars, amino acids, and organic acids used (Tab. 1). In contrast to strain Ac_23_E3^T, strain A22_HD_4H^T grew on pure agar. In order to confirm the ability of strain A22_HD_4H^T to endure starvation, the strain was incubated in bare SSE solution without the addition of an additional nutrient source. The strain endured 110 days of starvation (Suppl. Fig. S2). Strain Ac_23_E3^T grew on the polymer laminarin while strain A22_HD_4H^T did not show this feature. Like *B. fastidiosa* A2-16^T (Foesel *et al.*, 2013) strain Ac_23_E3^T showed the ability to grow on solid media containing the polymers starch, cellulose, and chitin while this feature was not present in strain A22_HD_4H^T (Tab. 1). In addition, the appearance of clearing zones after staining with Congo red verified the utilization of cellulose by Ac_23_E3^T. Strain A22_HD_4H^T showed no clearing zones after staining with Congo red. Furthermore, both strains revealed no decoloration of starch media after staining with Lugol´s solution. Exoenzyme profiles of strains A22_HD_4H^T and Ac_23_E3^T as determined with API ZYM test systems (Biomérieux) resembled each other (Supplementary Tab. S3). The main difference to *B. fastidiosa* A2-16^T is the expression of some sugar degrading enzymes like β-glucosidase and N-acetyl-β-glucosaminidase, in the two novel strains, which is in congruence with the range of growth substrates identified.

In summary, strains A22_HD_4H^T and Ac_23_E3^T are aerobic, chemoorganoheterotrophic, white to pink pigmented mesophiles with a broad pH and temperature tolerance. Common features of the two novel isolates and the earlier described *Acidobacteria* subdivision 4 isolates from soil, *B. fastidiosa* A2-16^T and *P. methylaliphatogenes* K22^T, are the preference for complex proteinaceous substrates, and the broad ranges of pH values and temperatures tolerated, albeit every strain shows its individual growth range according to the habitat derived from. *Candidatus* ‘C. thermophilum’ as a photoheterotroph from microbial mats differs due to its habitat preferences and primary metabolism. The different G+C content of genomic DNA and the fatty acids composition

distinguish the novel strains from their next phylogenetic relative *B. fastidiosa* A2-16^T. Beside the formation of cell chains, the results of DNA-DNA hybridization clearly differentiate strain Ac_23_E3^T and strain A22_HD_4H^T from each other. Additionally, the optimal growth temperature of strain Ac_23_E3^T (36-44°C) clearly exceeds the optimal growth temperature of strain A22_HD_4H^T (24-36°C). Based on phylogeny, morphology, and physiology of the strains A22_HD_4H^T and Ac_23_E3^T, the novel genus *Aridibacter*, including the two novel species, *A. famidurans* and *A. kavangonensis*, is proposed.

5.4. Description of *Aridibacter*, gen. nov.

Gram-negative, non-spore-forming, non motile rods that occur in single cells or short chains and divide by binary fission. Cytochrome *c*-oxidase-negative. Catalase-positive. No capsule formation. Aerobic, chemoorganotrophic, mesophiles. Low amounts of NaCl are growth enhancing. Major fatty acids $C_{15:0}$ iso, summed feature 1 ($C_{13:0}$ 3-OH/$C_{15:1}$ iso H), and summed feature 3 ($C_{16:1}$ ω7c/$C_{16:1}$ ω6c). Major quinone is MK-8. The type species is *Aridibacter famidurans*.

5.4.1. Description of *Aridibacter famidurans*, sp. nov.

Aridibacter famidurans (fa.mi.du'rans. L. fem. n. *fames* hunger; L. part. adj. *durans* the one who survives; N.L. part. adj. *famidurans* surviving hunger).

The description of *A. famidurans* complies with the description of the genus and in addition comprises the following characteristics. Cells are 2.5-3.0 µm long and 0.9 µm in diameter. Liquid cultures have a yellow to bright pink color. Dependent on the age of culture aggregates and flocks are formed even shaken cultures. Colonies on agar plates have a size of 0.1-0.2 mm are circular, pink in color, translucent, convex, and opaque with entire margins. Grows at temperatures of 15-44°C and pH 4.0-9.5. Under optimal growth conditions doubling time is 5.49 h. The strain grows at NaCl concentration up to 1% (w/v), best at a NaCl concentration of 0.25% (w/v).

The major fatty acids are $C_{15:0}$ iso, summed feature 3 ($C_{16:1}$ ω7c/$C_{16:1}$ ω6c), summed feature 1 ($C_{13:0}$ 3-OH/$C_{15:1}$ iso H), and $C_{17:0}$ anteiso.

Grows on rhamnose, xylose, N-acetylgalactosamine, protocatechuate, casamino acids, casein hydrolysate, peptone, yeast extract. On cellobiose, L-asparagine, heptanoic acid weak growth is detected. No growth is observed on arabinose, erythrose, erythrulose, fructose, fucose, galactose, glucose, lactose, lyxose, maltose, mannose, melizitose, raffinose, sorbose, sucrose, trehalose, glucosamine, N-acetylglucosamine, acetoin, adonitol, arabitol, dulcitol, lyxitol, mannitol, myo-inositol, sorbitol, xylitol, alanine, arginine, aspartate, cysteine,

glutamate, glycine, histidine, hydroxy-proline, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, threonine, tryptophan, tyrosine, valine, adipate, acetate, ascorbate, benzoate, trimethoxybenzoate, butyrate, α-hydroxybutyrate, β-hydroxybutyrate, γ-hydroxybutyrate, isobutyrate, caproate, caprylate, citrate, isocitrate, crotonate, formate, fumarate, gluconate, 2-oxogluconate, glucuronate, 2-oxoglutarate, glycolate, glyoxylate, isovalerate, laevulinate, lactate, malate, maleic acid, malonate, nicotinic acid, oxaloacetate, propionate, pyruvate, shikimate, succinate, tartrate, 2-oxovalerate, butanol, 1,2-butandiol, 2,3-butandiol, ethanol, ethylene glycol, glycerol, methanol, propanol, 1,2-propandiol, fermented rumen extract, laminarin, and Tween 80.

Enzyme activities are alkaline and acid phosphatase, leucine arylaminidase, valine arylaminidase, trypsin, β-glucosidase, and N-acetyl-β-glucosaminidase. Weak reactions of the enzymes α-glucosidase, α-chymotrypsin, esterase lipase C8, lipase C14, and naphtol-AS-BI-phosphohydrolase are detected. No activities of the enzymes esterase C4, cysteine arylaminidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-mannosidase, and α-fucosidase are determined. Aesculin and 4-nitrophenyl- β-D-galactopyranoside are hydrolyzed. Gelatine is hydrolyzed after one week. Urease, arginine dihydrolase, and indol production negative. The DNA G+C content is 53.2 mol%. The type strain is A22_HD_4H^T (= DSM 26555^T = LMG27985^T), and was isolated from a sandy subtropical savanna soil in Erichsfelde, Namibia.

5.4.2. Description of *Aridibacter kavangonensis*, sp. nov.

Aridibacter kavangonensis (ka.van.go.nen´sis N.L. masc. adj. *kavangonensis* deriving from the Kavango region, Namibia).

The description of *A. kavangonensis* complies with the description of the genus and the following additional characteristics. Cells are 2.5-3.0 μm long and 0.6-0.7 μm in diameter and form chains of 2-4 cells. Colonies have a size of 0.2-0.3 mm, are circular, white with a bright pink hue, translucent, convex, and opaque with entire margins. No aggregates are formed in liquid culture. Grows from 12-47°C and pH 3.5-10.0. Doubling time under optimal growth conditions is 6.1 h. The strain grows at NaCl concentrations of up to 1% (w/v), and best at a NaCl concentration of 0.25% (w/v).

The major fatty acids are $C_{15:0}$ iso, summed feature 3 ($C_{16:1}$ ω7c/$C_{16:1}$ ω6c), summed feature 1 ($C_{13:0}$ 3-OH/$C_{15:1}$ iso H), and $C_{17:0}$ anteiso.

Grows on maltose, rhamnose, N-acetylgalactosamine, fumarate, isovalerate, protocatechuate, casamino acids, casein hydrolysate, peptone, yeast extract, laminarin, cellulose. On lactose, glutamine, L-asparagine, trimethoxybenzoate, starch, and chitin weak

growth is detected. No growth is observed on arabinose, cellobiose, erythrose, erythrulose, fructose, fucose, galactose, glucose, lyxose, mannose, melizitose, raffinose, sorbose, sucrose, trehalose, xylose, glucosamine, N-acetylglucosamine, acetoin, adonitol, arabitol, dulcitol, lyxitol, mannitol, myo-inositol, sorbitol, xylitol, alanine, arginine, aspartate, cysteine, glutamate, glycine, histidine, hydroxy-proline, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, threonine, tryptophan, tyrosine, valine, adipate, acetate, ascorbate, benzoate, butyrate, heptanoic acid, α-hydroxybutyrate, β-hydroxybutyrate, γ-hydroxybutyrate, isobutyrate, caproate, caprylate, citrate, isocitrate, crotonate, formate, gluconate, 2-oxogluconate, glucuronate, 2-oxoglutarate, glycolate, glyoxylate, laevulinate, lactate, malate, maleic acid, malonate, nicotinic acid, oxaloacetate, propionate, pyruvate, shikimate, succinate, tartrate, 2-oxovalerate, butanol, 1,2-butandiol, 2,3-butandiol, ethanol, ethylene glycol, glycerol, methanol, propanol, 1,2-propandiol, fermented rumen extract, and Tween 80.

Enzyme activities comprise alkaline and acid phosphatase, leucine arylaminidase, valine arylaminidase, trypsin, α-chymotrypsin, naphtol-AS-BI-phosphohydrolase, β-glucosidase, N-acetyl-β-glucosaminidase. Weak reactions of esterase lipase C8 and β-glucuronidase are detected. No activity of esterase C4, lipase C14, cysteine arylaminidase, α-galactosidase, β-galactosidase, α-glucosidase, α-mannosidase, and α-fucosidase are present. Aesculin and 4-nitrophenyl- β-D-galactopyranoside are hydrolyzed. Gelatinase, urease, indol production, and arginine dihydrolase negative. The DNA G+C content is 52.6 mol%. The type strain is Ac_23_E3^{T} (= DSM 26558^{T} = LMG 27597^{T}), which was isolated from a sandy subtropical savanna soil in Mashare, Namibia.

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5.7. Supplementary Tables

Supplementary Table S1. Full cellular fatty acid profile of the strains A22_HD_4H^T and Ac_23_E3^T. Percentages of total fatty acids are outlined in the table. Summed features are two or more fatty acids which were not separated by MIDI chromatograph.

Fatty acid	A22_HD_4H^T	Ac_23_E3^T
Saturated		
12:0	0.35	0.24
14:0	0.47	0.67
16:0	**3.63**	**4.96**
18:0	0.23	-
20:0	1.05	0.48
Unsaturated		
14:1 ω5c	1.85	0.77
18:3 ω6c	-	0.46
Methyl-branched		
11:0 iso	0.39	0.79
13:0 iso	**4.14**	**3.45**
14:0 iso	0.24	-
15:0 iso	**35.14**	**38.01**
15:1 anteiso A	0.70	0.33
16:0 iso	0.86	1.90
16:0 anteiso	0.47	0.61
16:1 iso G	0.33	-
17:0 iso	0.61	1.67
17:0 anteiso	**6.19**	**10.85**
17:1 iso ω9c	0.66	2.30
17:1 anteiso A	3.01	**4.11**
Hydroxy		
12:0 3-OH	0.36	-
13:0 iso 3-OH	0.29	-
17:0 iso 3-OH	1.80	1.47
Summed Feature		
1 (15:1 iso H/13:0 3-OH)	**17.15**	**11.65**
3 (16:1 ω7c/16:1 ω6c)	**20.05**	**15.27**

Supplementary Table S2. Single substrate concentration used for determination of substrate range of the strains A22_HD_4H^T and Ac_23_E3^T in liquid culture.

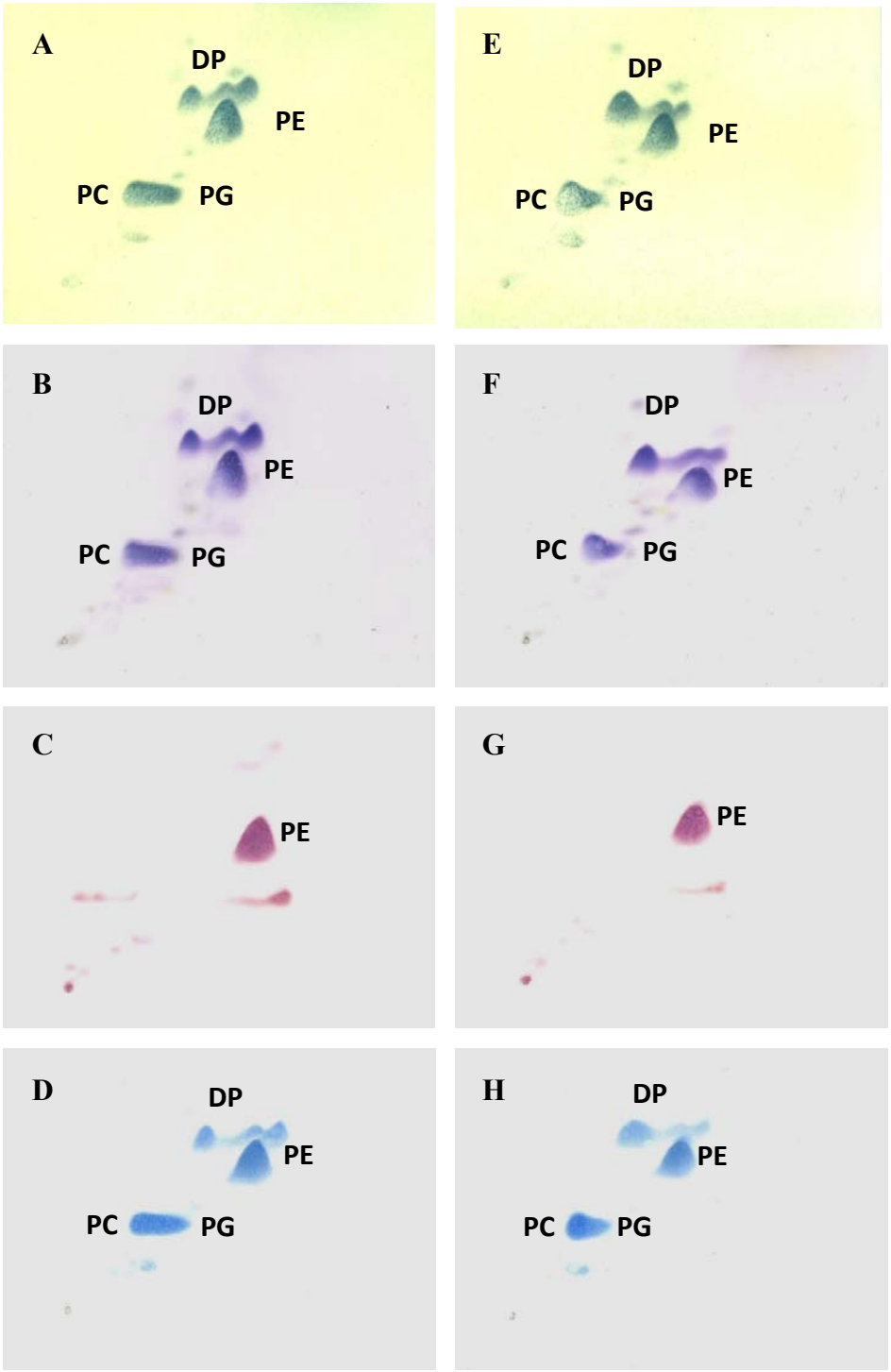
Substrate	Concentration [mM]	Substrate	Concentration [mM]
Arabinose	5	Glycine	5
Cellobiose	5	Histidine	5
Erythrose	5	Hydroxy-Proline	5
Erythrulose	5	Isoleucine	2
Fructose	5	Leucine	5
Fucose	5	Lysine	5
Galactose	5	Methionine	5
Glucose	5	Ornithine	2
Lactose	5	Phenylalanine	5
Lyxose	5	Proline	2
Maltose	5	Threonine	5
Mannose	5	Tryptophan	1.25
Melizitose	5	Tyrosine	5
Raffinose	5	Valine	5
Rhamnose	5	Adipate	5
Sorbose	5	Acetate	5
Sucrose	5	Ascorbate	5
Trehalose	5	Benzoate	5
Xylose	5	Trimethoxybenzoate	5
Glucosamine	5	Butyrate	2.5
N-acetylglucosamine	5	α-Hydroxybutyrate	2.5
N-acetylgalactosamine	5	β-Hydroxybutyrate	2.5
Acetoin	5	γ-Hydroxybutyrate	2.5
Adonitol	5	Isobutyrate	2.5
Arabitol	10	Caproate	5
Dulcitol	5	Caprylate	5
Lyxitol	5	Citrate	2
Mannitol	5	Isocitrate	5
Myo-Inositol	5	Crotonate	5
Sorbitol	5	Formate	2.5
Xylitol	5	Fumarate	5
Alanine	5	Gluconate	5
Arginine	5	2-Oxogluconate	5
Asparagine	2	Glucuronate	5
Aspartate	2	2-Oxoglutarate	5
Cysteine	2	Glycolate	5
Glutamate	2	Glyoxylate	5
Glutamine	2	Heptanoic acid	5
Isovalerate	0.5	Ethylene glycol	5

Laevulinate	5	Glycerol	5
Lactate	2	Methanol	2
Malate	5	Propanol	5
Maleic acid	5	1,2-Propandiol	5
Malonate	5	Fermented rumen extract	5
Nicotinic acid	2	**Substrate**	**Concentration [% w/v]**
Oxaloacetate	5	Laminarin	0.05
Propionate	5	Tween 80	0.001
Protocatechuate	5	Casamino acids	0.05
Pyruvate	10	Casein hydrolysate	0.05
Shikimate	5	Peptone	0.05
Succinate	10	Yeast extract	0.05
Tartrate	2	**Substrate**	**Concentration [mg l^{-1}]**
2-Oxovalerate	5	Starch	500
Butanol	5	Cellulose	500
1,2-Butandiol	5	Chitin	500
2,3-Butandiol	5	Avicel	500
Ethanol	5		

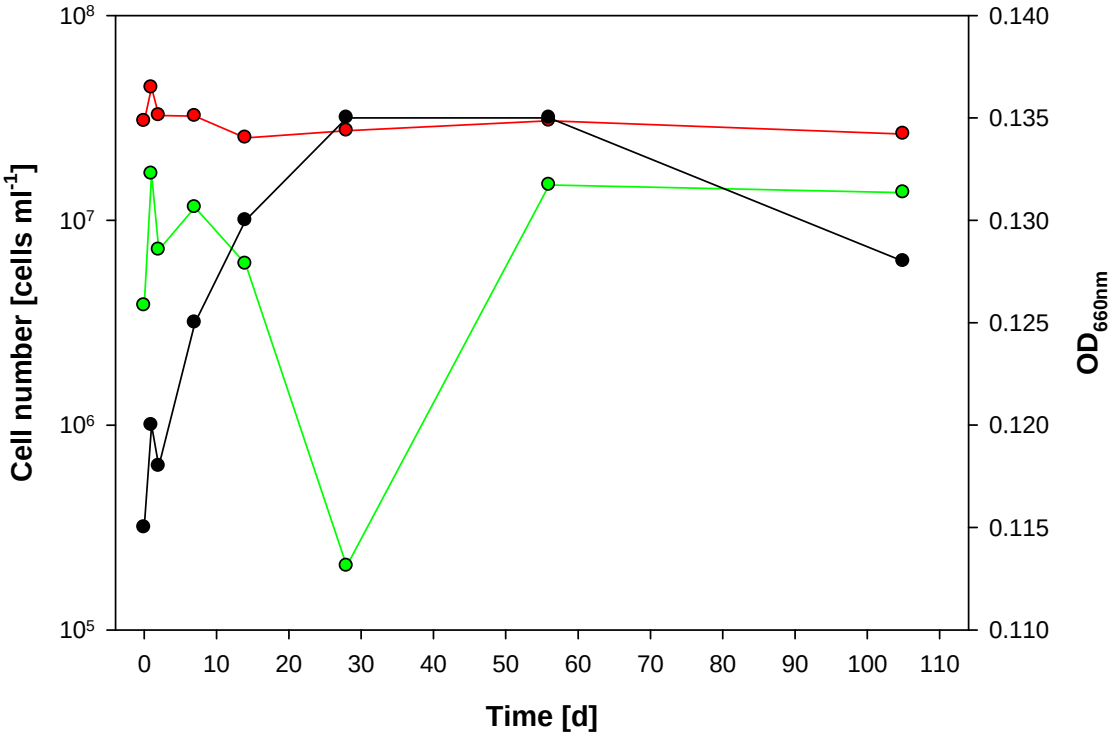
Supplementary Table S3. Enzyme activities of A22_HD_4H^T and Ac_23_E3^T.
+, positive; -, negative; (+), weak enzyme activity detected.

Enzyme	A22_HD_4H^T	Ac_23_E3^T
Alkaline phosphatase	+	+
Acid phosphatase	+	+
Naphtol-AS-BI-phosphohydrolase	(+)	+
Leucine arylaminidase	+	+
Valine arylaminidase	+	+
Cysteine arylaminidase	-	-
Arginine dihydrolase	-	-
Indol production	-	-
Esterase lipase C8	(+)	(+)
Lipase C14	(+)	(+)
Esterase C4	-	-
Trypsin	+	+
α-Chymotrypsin	(+)	+
Urease	-	-
Gelatinase	(+)	-
α-glucosidase	(+)	-
β-glucosidase	+	+
α-mannosidase	-	-
α-galactosidase	-	-
β-galactosidase (API ZYM)	-	-
β-galactosidase (API 20 NE)	+	+
α-fucosidase	-	-
β-glucuronidase	-	-
N-acetyl-β-glucosaminidase	+	+

5.8. Supplementary Figures



Supplementary Figure S1. Polar lipids composition of the strains A22_HD_4H^T (**A-D**) and Ac_23_E3^T (**E-H**) on thin layer chromatography. Staining for determination of the polar lipids with dodecamolydophosphoric acid (**A, E**), ninhydrin (**B, F**), anisaldehyd sulfuric acid (**C, G**), Zinzadze reagent (**G, H**). For lipid separation chloroform:methanol:water (65:25:4, v/v/v) was used in the first direction and chloroform:methanol:acetic acid:water (80:12:15:4, v/v/v/v) in the second direction. PE: phosphatidylethanolamine, PC: phosphatidylcholine, DPG: diphosphatidylglycerol, PG: phosphatidylglycerol.



Supplementary Figure S2: Starvation experiment: Strain A22_HD_4H^T was incubated for 110 days in pure SSE. Green, colony forming units; red, total cell numbers; black, OD_{660nm}.

Chapter 6

General Discussion

6.1. The role of microorganisms in the nutrient cycling of subtropical savannah soils

Active soil microorganisms like *Actinobacteria* and *Firmicutes* (Janssen, 2006) as detected in the Mashare sand soils (Chapter 5) degrade organic biomass, plant and animal residues to remineralize carbon, nitrogen and phosphorus (Clinton *et al.*, 2010) by exoenzyme activities (Chapter 3) and nitrogen transformations (Chapter 4). In the examined subtropical savannah soils the degradation potential of carbon compounds exceeded the degradation potential of complex phosphorus and nitrogen compounds. However, the degradation of complex nitrogen compounds and the subsequent transformation by ammonification and nitrification positively correlated to each other and provided ammonium and nitrogen even in low fertile soils. Hence, in the present study, the determination of microbial activity patterns in Namibian and Angolan arenosols facilitate the elucidation of nutrient cycling, soil fertility and adequate nutrient supply of animals, plants and microorganisms in nutrient limited arenosols.

6.2. Impact of environmental parameters on microbial activity

The microbial activity, the composition of the soil microbial community and thereby nutrient cycling are directly effected by several (a)biotic factors (Carreiro *et al.*, 2000; Treseder *et al.*, 2012) like soil organic matter, nutrient and water content, temperature, form of litter, pH, land use type and cultivation (Baldrian *et al.*, 2013; Rinkes *et al.*, 2013; Waldrop *et al.*, 2000). In the present study several Namibian and Angolan arenosols samples differing in soil type, land use type and water availability were investigated. The aim of this sampling strategy was the identification of the main environmental factors controlling microbial activity in nutrient limited savannah soils. Here, soil type, land use type and water availability were identified as essential environmental parameter controlling the microbial activity and the nutrient cycling in nutrient limited subtropical savannah soils.

6.2.1. Soil type

Previous studies showed that microbial activity is positively correlated to soil organic matter content (Sinsabaugh *et al.*, 2008). In the present work exoenzyme activity measurements obtained from soils with varying soil organic matter (SOM) contents confirmed this correlation (Chapter 3). Highest activity values of the β-glucosidase, β-xylosidase, phosphatase and aminopeptidase were determined in dark loamy sand soils of Mutompo, in

the old flood plain soils of Mashare and in the peatland soils of Cusseque. SOM contents of these soils stimulate the inducible enzymes β-glucosidase, β-xylosidase and phosphatase resulting in high exoenzyme activity values (Chrost, 1991; Sinsabaugh *et al.,* 1992). Additionally, the high input of organic matter in the form of leaves, litter and roots within the dense vegetation of the riparian woodland and bushveld savannah soils and of the Angolan horticulture soil increases the inducing effect. SOM provides nutrients and water (Wang *et al.*, 2003; Schlesinger *et al.*, 2000; Cleveland *et al.*, 2007) and stable aggregates (Kandeler *et al.*, 1999a; Kanazawa *et al.*, 1986). It preserves the microbial community against drought and starvation. Hence, total cell numbers in the Angolan and Namibian soils and thereby activities reached highest numbers (Manzoni *et al.*, 2012). Additionally, the binding of exoenzymes to biomass (Schlosser *et al.*, 1997; Valásková *et al.*, 2006), to abiotic soil particles (George *et al.*, 2005; Nannipieri *et al.*, 1996) or clay (Boyd & Mortland, 1990) prevents the enzymes which are located in the interphase between water and solid soil phase (Norde *et al.*, 1991) from degradation or leaching (Naidja *et al.*, 2000). Therefore, SOM content lead to high activity values of exoenzymes even in nutrient limited Namibian and Angolan arenosols.

6.2.2. Land use type

Microbial activities show fast response to external disturbances (Dick, 1994), i. e. fertilization (Lovell *et al.*, 1995), burning, thinning, ploughing or combination (Dick, 1988; Concilio *et al.*, 2006; Gupta *et al.*, 1988) heavy metals or organic xenobiotics (Kandeler *et al.*, 1999b; Baldrian *et al.*, 2009). In soils with a low impact of human activities like the riparian woodland and bushveld savannah soils exoenzyme activities and nitrogen turnover rates of the microbial community reached highest values (Dick, 1984; Dick, 1988; Frank *et al.*, 2006). Riparian woodland and bushveld savannah soils provide increased SOM content and thereby nutrients, water (Wang *et al.*, 2003; Schlesinger *et al.*, 2000; Cleveland *et al.*, 2007), aggregate stability (Kandeler *et al.*, 1999a; Kanazawa *et al.*, 1986) for the soil microbial community. Pristine soils have a dense vegetation and high organic input in the form of litter, roots and plant residues (Bolton, 1985; Martens *et al.*, 1992; Goyal *et al.*, 1993). Furthermore, the presence of roots restrains water and nutrients by adhesion forces and stimulates the soil microbial community by rhizodeposition (Hinsinger *et al.*, 2009; Jones *et al.*, 2009). Therefore, the microbial community of riparian woodland and bushveld savannah soils inhabits optimized conditions even in arenosols and thereby reached highest activity values. However, the activities of the soil microbial community decreased with increasing anthropogenic impact. Agriculturally used fields obtained lowest exoenzyme activity values and nitrogen turnover rates in the present study since they are disturbed by overexploitation

and alteration of bulk soil density and soil structure (Johnson *et al.*, 1991). Fertilization in the irrigated fields of the Okavango basin causes acidification of soils (Goyal *et al.*, 1993), alters the ionisation and solubility of enzymes, substrates, cofactors, stability of protein structures, organic xenobiotics, and metals (Kandeler *et al.*, 1999b; Baldrian *et al.*, 2009). Hence, microbial activity values in the agriculturally used soils were decreased.

6.2.3. Water availability

In the present study, the microbial activities and consequently nutrient cycling were effected by the soil water content. Water availability positively influences microbial activity in sandy, in loamy soils (Schjønning *et al.*, 2011) and even in compost (Liang *et al.*, 2003). Former studies confirmed that CO_2 flux (Davidson *et al.*, 1998) as a microbial activity indicator increases with increasing water content. A total water saturation of the soils causes anoxic conditions probably resulting in a decrease of total bacterial cell numbers and activity values. Due to low water retention capacities in the majority of the examined soils, this effect could be neglected for the arenosols but had to be considered for the peatland soils of Cusseque. In the investigated soils total cell numbers, exoenzyme activities and nitrogen turnover rates decreased after the dry season. The drought caused low water availability and inhibited microbial activity by lowering the intracellular water potential, by restricting substrate supply (Stark *et al.*, 1995) and inhibiting substrate enzyme interactivity.

Furthermore, the water stress probably lysed the majority of the microbial cells, reduced the bacterial community or decreased the activity levels by the formation of duration form like spores. Only microorganisms which were adapted to water stress and heat by sporulation or resistant vegetative cells survived the drought. However, the increased SOM in the pristine soils prevented the microbial community by the provision of water and nutrients to the soil microbial community and resulted in high activity values.

6.3. Composition of the active microbial community in Namibian arenosols

In arenosols microorganisms are exposed to extreme environmental conditions like water stress, heat, leaching and limited nutrient supply. Hence, only microorganisms adapted to nutrient limitation and water stress survive the challenging conditions of the subtropical savannah soils. The stimulation experiment (Chapter 5) revealed *Actinobacteria*, *Proteobacteria* and *Firmicutes* as the most abundant active bacterial phyla in the Mashare soils. Representatives of these phyla are perfectly adapted to the conditions in the arenosols by the formation of spores (*Bacillus, Paenibacillus, Sporosarcina*) (Chen *et al.*, 2014), spore like vegetative cells (*Arthrobacter crystallopoietes*) (Boylen, 1973), DNA reparation systems,

accumulation of manganese supporting DNA reparation systems (*Enterococcus, Arthrobacter*) (Mongodin *et al.*, 2006), variation of cell cycle from rods to coccoid cells for reduction of volume to surface relation (*Arthrobacter*) (Ensign, 1970) or additional plasmids for the degradation of pollutants and soil organic matter (*Arthrobacter*) (Mongodin *et al.*, 2006).

Beside the adaptation to water stress and heat, the prevailing phyla were also adapted to the nutrient limitation in the examined arenosols. Filamentous growth of *Actinobacteria* (Rinkes *et al.*, 2013; Větrovský *et al.*, 2014) and additional plasmids encoding for the degradation of complex compounds and even pollutants (*Arthrobacter*) (Hagedorn *et al.*, 1975; Mongodin *et al.*, 2006) may enable the usage of SOM as nutrient source in the riparian woodland and bushveld savannah soils of Mashare. Different species of the *Rhizobiales, Paenibacillaceae*, *Rubrobacter* and *Arthrobacter* (Sellstedt *et al.*, 2013) fix nitrogen directly from air and endure nitrogen limitation in the examined soils. In anaerobic soil microhabitats some *Arthrobacter* (Eschbach *et al.*, 2003) and *Exiguobacterium* (Vishnivetskaya *et al.*, 2009) species probably use nitrate as terminal electron acceptor. The genome of the thermophilic *Exiguobacterium* sp. AT1b contains four different nitrate reductase genes (Vishnivetskaya *et al.*, 2009) and genes for cellulase and hemicellulase degradation (Vishnivetskaya *et al.*, 2011). Soil physicochemical analysis (Chapter 3) revealed phosphorus limitation in the Namibian subtropical savannah soils. The *Arthrobacter* genome contains 25 genes encoding for resistance to heavy metals or stresses and four genes encoding RNA polymerase r70 factor, which is involved in the phosphate starvation response (Makino *et al.*, 1993; Mongodin *et al.*, 2006). Interestingly, *Enterococcus* and *Enterobacteriales* probably deriving from wild animals and cattle prevailed the activity abundances of the *Firmicutes* and *Proteobacteria* in the riparian woodland and bushveld savannah soils after the drought. Fast growing microorganisms with a high demand of nitrogen and phosphorus like *Proteobacteria* (Gusewell *et al.*, 2005) benefit from the addition of the nutrients and rewetting, while other bacterial species enduring in inactive cell forms were unable to react to the nutrient addition pulse in a short time. Additionally, *Enterococcus* and *Enterobacteriales* showed low abundance levels in the irrigated fields which were surrounded by a fence and protected against wild animals and grazing cattle. Moreover, *Enterobacteriales* are badly adapted to high concentrations of metals and fertilizers in irrigated soils in contrast to *Firmicutes*, like *Exiguobacterium. Exiguobacterium* predominantly known from permafrost soils is also abundant in tropical soils (Rodrigues *et al.*, 2007), halotolerant, radioresistant (Vishnivetskaya *et al.*, 2009), tolerates broad temperature and pH ranges (Vishnivetskaya *et al.*, 2009) and

grows despite heavy metal stress. The presence of additional plasmids probably encoding for the degradation of SOM or metal stress response explains the high activity values of *Exiguobacterium* in irrigated fields.

Although *Acidobacteria* constitute up to 77% of the soil microbial community (Janssen, 2006) with subdivision 6 *Acidobacteria* as the most abundant representatives of this phylum (George *et al.*, 2011), only few information about their role in the environment is available. Despite low activity abundances in the stimulated Mashare soils, new insights into their function and controlling mechanisms were gained. Both the land use type and the water availability effected the composition of the *Acidobacteria* phylum. Subdivision 3, 6 and 16 *Acidobacteria* seem to be well adapted to the fertilization and maize exudates in the irrigated fields. Similar to *Arthrobacter* and *Exiguobacterium*, additional plasmids encoding for the degradation of pollutants or soil exudates might provide growth advantages of these subdivisions. In the woodland and bushveld savannah soils the correlation of high abundances of subdivision 6 *Acidobacteria* and high exoenzyme activities and nitrogen turnover rates indicate a possible important role in the nutrient cycling. However, subdivision 3 *Acidobacteria* badly survived dry conditions in the pristine sand soils after the prolonged drought in contrast to subdivision 4 *Acidobacteria* reaching high activity abundances in these soils. Subdivision 4 *Acidobacteria* seem to be well adapted to drought and nutrient limitation in subtropical savannah soils. Here, the presence of carotinoids as detected for *Blastocatella fastidiosa* (Foesel *et al.*, 2013), *Terriglobus roseus* (Eichorst *et al.*, 2007) and *Bryocella elongata* (Dedysh *et al.*, 2012) might counteract radical oxygen species caused by increased UV radiation and prevent the cells against DNA double strand breaks (Liu *et al.*, 2012). However, no capsule formation and sporulation were determined for representatives of this phylum so far. Therefore, other mechanisms are thought to be evolved in the adaptation mechanisms of drought and further investigations on this subphylum will be necessary.

6.4. Two novel representatives of subdivision 4 *Acidobacteria* isolated from Namibian arenosols

Acidobacteria constitute an abundant fraction in the soil microbial community (Janssen, 2006). Illumina high throughput sequencing of the nutrient stimulated Namibian soil samples revealed a low percentage of the *Acidobacteria* within the active soil microbial community. But within the *Acidobacteria* subdivision 4 *Acidobacteria* were prevailing. Therefore, subdivision 4 *Acidobacteria* seem to have an important role in subtropical savannah soils. In the present study two novel representatives of subdivision 4 *Acidobacteria* were isolated from subtropical savannah soils, physicochemically characterized and validly described.

Strain A22_HD_4H^T and strain Ac_23_E3^T were isolated from two different Namibian arenosols and belong to subdivision 4 *Acidobacteria*. Members of this phylum are ubiquitous in different habitats and perform different metabolisms (Janssen, 2006). The only known photosynthesis performing *Acidobacterium* so far '*Candidatus* C. thermophilum' belongs to subdivision 4 *Acidobacteria* (Bryant *et al.*, 2007). Moreover, representatives of this subdivision utilize complex compounds, accumulate carotinoids for UV radiation defense and tolerate broad temperature and pH ranges (Foesel *et al.*, 2013; Huber *et al.*, 2014; Crowe *et al.*, 2014). Based on a 16S rRNA gene identity of about 93% with their next validly described relative *Blastocatella fastidiosa* the new genus *Aridibacter* with the two new species *A .famidurans* and *A. kavangonensis* were proposed. Despite a 16S rRNA gene identity of 97.3% of the two new isolates to each other, DNA-DNA hybridization revealed the presence of two new species.

A. famidurans and *A. kavangonensis* probably take an important part in the carbon cycling. Both species express β-glucosidase and N-acetyl-β-glucosaminidase. Furthermore, *A. kavangonensis* is able to degrade cellulose and grows on starch, while *A. famidurans* is adapted to extreme nutrient limitation and even grows on pure agar.

The subdivision 4 *Acidobacteria* prevailed in nutrient limited savannah soils even after prolonged drought. Therefore, representatives of the subdivision have evolved adaptation mechanisms against water stress and starvation. However, both *Aridibacter* species, *Blastocatella* and *Pyrinomonas* as the only described subdivision 4 *Acidobacteria* form no capsule or spores (Foesel *et al.*, 2013; Huber *et al.*, 2014; Crowe *et al.*, 2014). However, thickened cell walls (about 30 nm) probably protect the starvation specialist *A. famidurans* from starvation and water stress. This mechanisms would explain the survival of *A. famidurans* for two years in a nutrient limited SSE medium and in its natural habitat - the dry subtropical savannah soils of Namibia, respectively.

The present study demonstrated that the activity and the composition of the microbial community in arenosols is effected by soil type, land use type and water availability. Moreover, microorganisms preserve soil fertility even in nutrient poor soils by the degradation of soil organic matter and the remineralization of the major nutrients carbon, nitrogen and phosphorus.

6.5. References

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